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SVEUČILIŠTE U SPLITU
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Igor Borić

MULTIDISCIPLINARNI PRISTUP U OBRADI KOŠTANIH OSTATAKA EKSHUMIRANIH IZ MASOVNIH GROBNICA

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Doktorska disertacija sadrži rezultate znanstvenih istraživanja provedenih na Katedri za sudsku medicinu Medicinskog fakulteta Sveučilišta u Splitu.

VODITELJ RADA: prof. dr. sc. Davorka Sutlović

ZAHVALJUJEM

Mojim roditeljima,
koji su me usmjerili i školovali
Davorki,
koja me poticala i pomagala,
bez koje ne bi bilo ovog rada
Ani i svima,
koji su pomogli
Maji,
bez koje ne bi bilo ničega.

Rad posvećujem Udruzi „Daksa“ i svim žrtvama u masovnim grobnicama koje nikad neće biti identificirane.

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1. POPIS OZNAKA I KRATICA

DNK	deoksiribonuleinska kiselina (engl. DNA *Deoxyribonucleide Acid*)
BMC	mineralni sadržaj kosti (engl. *Bone Mineral Content*)
BMD	mineralna gustoća kosti (engl. *Bone Mineral Density*)
DXA	denzitometrija (engl. *Dual –energy X-ray Absorptionmetry*)
AAS	atomska apsorpcijska spektrometrija (engl. *Atomic Apsorption Spectroscopy*)
FAAS	plamena atomska apsorpcijska spektrometrija (engl. *Flame Atomic Apsorption Spectroscopy*)
UV VIS	apsorpcijska spektrometrija ultraljubičastim i vidljivim svjetlom (engl *Ultraviolet and visible Apsorption Spectroscopy*
Ca	kalcij (engl. *Calcium*)
P	fosfor (engl. *Phosphorus*)
L	lijevo (eng. *Left*)
R	desno (engl. *Right*)

2. PREGLED OBJEDINJENIH RADOVA

Doktorska disertacija nastala je objedinjenjem triju znanstvenih članaka:

1. Borić I, Ljubković J, Sutlović D. Discovering the 60 years old secret: Identification of the World War II mass grave victims from the island of Daksa near Dubrovnik, Croatia. *Croat Med J* 2011; **52**:327-35.

2. *Sutlović D, *Borić I, Zulim T, Vučinović A. Identification process of skeletal remains from mass graves: our experience and proposal guidelines. *Legal Medicine* 2015; **17**:102-108.

3. *Sutlović D, *Borić I, Slišković L, Popović M, Knezović Z, Nikolić I, Vučinović A, Vučinović Z. Bone mineral density of skeletal remains: Discordant results between chemical analysis and DXA method. *Legal Medicine* 2016; **20**:18-22.

* Oba autora imaju jednak doprinos

2.1. UVOD

Identifikacija predstavlja utvrđivanje istinitosti osoba, dijelova tijela, tragova i predmeta u cilju otkrivanja pojedinih obilježja, koja omogućuju nesumnjivo i nesporno prepoznavanje. Proces identifikacije temelji se na dva osnovna postupka: 1. traženje i bilježenje karakterističnih osobina objekta ispitivanja i 2. uspoređivanje ustanovljenih značajki s već ranije poznatim podatcima o objektu za koji se identitet pretpostavlja, posebice prilikom istraživanja masovnih grobnica (1). Kod obrade masovnih grobnica iz Drugog svjetskog rata glavne probleme čine oskudni, često nedostatni ili nepostojeći antemortalni podatci, te visok stupanj degradacije deoksiribonukleinske kiseline (DNK), zbog protoka vremena i okolišnih uvjeta. Publicirani su brojni radovi u kojima su prikazani problemi identifikacije žrtava, nađenih u masovnim grobnicama poslije Domovinskog rata, Drugog svjetskog rata i Prvog svjetskog rata (2-5). Uspjeh sudske identifikacije u velikoj mjeri ovisi o opsegu i očuvanosti materijala koji je prikupljen na terenu (3,6). U svrhu prikrivanja ratnih zločina, odnosno prikrivanja mjesta ukopa tijela, zaraćene strane koriste se različitim metodama; iskapanje i premještanje ostataka iz jednog u drugo ukopno mjesto, daljnje premještanje na tercijarne lokacije, rastavljanje i miješanje dijelova tijela, stješnjavanje i drobljenje. To dodatno otežava ili onemogućava određivanja broja tijela, njihovo sastavljanje i identifikaciju, a broj fragmentiranih uzoraka koštanih ostataka vrtoglavo raste. Cijene DNK analize u takvim situacijama dosegnule bi visoke iznose, te ih realno nije moguće sve analizirati. Kako se, uz navedeno, u masovnim grobnicama često nalazi velik broj žrtava, relativno su rijetke idealne situacije s očuvanim lubanjama i pripadajućim zubima, te ostalim kostima u anatomskom položaju (2,5,7). U procesu prepoznavanja nužna je suradnja svih relevantnih stručnjaka, a metodama identifikacije potrebno je služiti se planski, primjenjujući različite metode koje će međusobnim upotpunjavanjem povećati uspješnost identifikacije i postići osnovni cilj: iskazati poštovanja prema preminulima vraćanjem njihovih ostataka obiteljima, čime se štite ljudska prava živih i umrlih (8,9). Treba imati na umu da se takvi koštani uzorci potencijalno mogu koristiti i u svrhu drugih znanstvenih istraživanja. Korištenjem antropoloških metoda utvrđuje se jesu li pronađeni ostatci ljudski i koliko je osoba u prikupljenim uzorcima. Za svaku osobu treba odrediti spol, dob u trenutku smrti, visinu, rasnu

pripadnost, postojanje traume ili patoloških stanja, uzrok smrti, te konačno - identitet. Ako identitet nije utvrđen primjenom standardnih metoda identifikacije, potrebno je primijeniti DNK-analizu (1). Primjena DNK analiza zauzima sve veću ulogu u postupcima identifikacije osoba. Jedno od važnijih dostignuća primjene molekularne biologije u sudskoj medicini je određivanje identiteta osoba DNK tipizacijom iz bioloških uzoraka. Posebno se to odnosi na identifikaciju pomoću uzoraka kosti ili zuba ekshumiranih tijela, koja se ne može obaviti drugim klasičnim metodama. Analiza DNK predstavlja vrlo korisnu i danas najtočniju metodu identifikacije u takvim slučajevima. Osnova analize je uspoređivanje DNK, izolirane iz uzoraka skeletnih ostataka (kosti ili zuba), s DNK izoliranom iz uzoraka krvi, brisa bukalne sluznice, dlake i sl. pretpostavljene najuže rodbine (2). Ipak, uloga klasičnih metoda u svakodnevnoj praksi još uvijek je neupitna, a glavni razlog tomu je njihova cijena. Pristupačnost postojećih klasičnih metoda optimalno je povezati s novim metodama, u svrhu što uspješnije identifikacije. U ovom radu ispitana je iskoristivost i mogućnosti dostupnih i ne odviše skupih antropometrijsko-fizikalnih analiza u procesu smanjenja broja uzoraka, tj. formiranja parova fragmenata lijevih i desnih natkoljeničnih kostiju iz kojih bi se napravila DNK analiza. Zbog navedenih posebnosti masovnih grobnica, posebice „starijih“, organizaciji rada i uzimanju uzoraka potrebno je pristupiti minuciozno. Uzevši u obzir realna financijska, vremenska i kadrovska ograničenja, kao i činjenicu da se masovne grobnice uglavnom nalaze na teško pristupačnim mjestima, postoji nezanemarivi rizik da se uzorci trajno i nepovratno oštete ili unište. Stoga je jedan od glavnih ciljeva ove studije bio uspostavljanje algoritma postupaka kojima bi se preveniralo nastajanje takvih problema i stvorio standard cjelovitog pristupa masovnoj grobnici. Naime, u do sada publiciranim radovima autori su uglavnom opisivali poteškoće vezane uz izolaciju DNK iz takvih uzoraka, dok se mali broj bavio odabirom broja i vrste koštanih uzoraka za DNK analizu (2,10-13). Prema dostupnoj literaturi, rezultati dobiveni u ovoj studiji predstavljaju prve objavljene publikacije usporedbe rezultata denzitometrije (mineralna gustoća kosti - BMD) s rezultatima koncentracija kalcija i fosfora, izmjerenih kemijskim metodama na koštanim uzorcima iz masovne grobnice.

2.1.1. CILJEVI ISTRAŽIVANJA

Vodeći računa o ranije navedenim ograničenjima i problemima prilikom obrade koštanih ostataka ekshumiranih iz masovnih grobnica, prvi i glavni cilj ovog istraživanja bio je dati prijedlog Algoritma za postupanje i obradu koštanih uzoraka ekshumiranih iz masovnih grobnica.

Za ostvarenje glavnog cilja postavljeni su podciljevi:

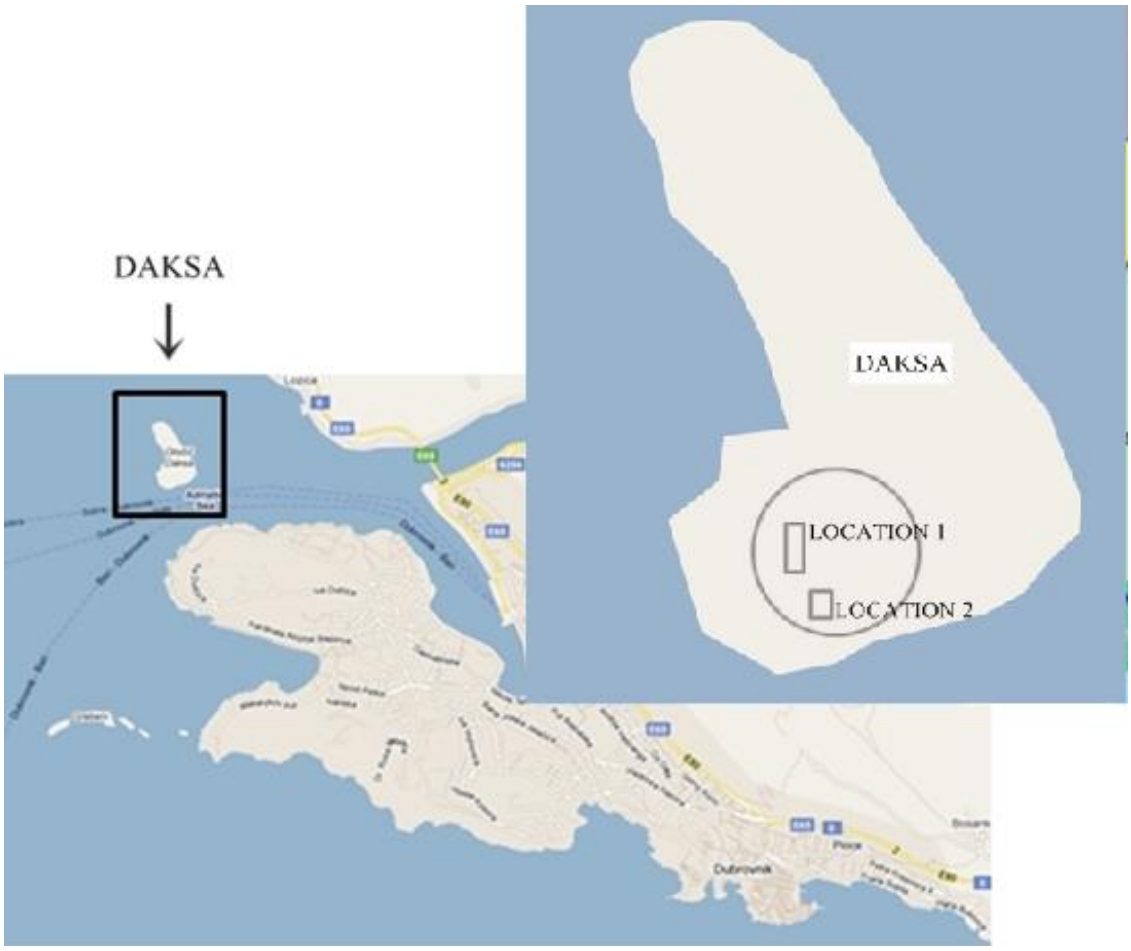
1. Predložiti prvi algoritam po kojem će se provesti cijelo istraživanje.
2. Istražiti mogućnosti redukcije broja uzoraka potrebnih za DNK analizu korištenjem različitih metoda antropoloških i fizikalnih mjerenja bedrenih kostiju, odnosno parcijalnih uzoraka (14).
3. Odrediti maseni udio minerala kalcija i fosfora u uzorcima kostiju iz vremena završetka II. Svjetskog rata, te ispitati pozitivnu korelaciju između masenog udjela kalcija i fosfora određenog kemijskom metodom i površinske gustoće kostiju izmjerene denzitometrijskom metodom za sve odabrane parove (fragmenti lijeve i desne bedrene kosti koje pripadaju istoj osobi) (15).
4. Statistički izraziti podudarnost između koštanih fragmenata za sve uspješno uparene koštane fragmente kao mjerilo uspjeha fizikalno-kemijskih analiza u svrhu formiranja parova.
5. DNK analizom dokazati opravdanost primjene antropoloških i fizikalnih mjerenja bedrenih kostiju iz reduciranih uzoraka, tj. dokazati da uzorci kostiju upareni fizikalnim metodama pripadaju istoj osobi.
6. Koristeći spoznaje proizašle iz svih rezultata napravljenih analiza, predložiti poboljšani konačni algoritam.

U radu je testirana hipoteza:

Antropometrijskim i fizikalnim mjerenjima fragmenata bedrene kosti moguće je reducirati broj uzoraka ekshumiranih iz masovnih grobnica potrebnih za DNK analizu.

2.2. MATERIJAL I METODE

Istraživanje je obuhvatilo analizu posmrtnih ostataka žrtava, Dubrovčana koji su strijeljani i sahranjeni na nenaseljenom otočiću Daksi pokraj Dubrovnika (Slika 1).



Slika 1. Smještaj otoka Daksa i položaj dvije masovne grobnice, Lokacija 1 i Lokacija 2.

Ekshumacija posmrtnih ostataka žrtava na Daksi je započela 24. rujna 2009. godine, a završena dana 29. rujna 2009. godine. Na lokaciji (odnosno na dvije mikrolokacije, označne kao 1 i 2) pronađeno je približno 10 000 uzoraka kostiju i fragmenata kostiju. Posebna pozornost posvećena je obradi 104 uzorka femura, uz pretpostavku da su se prema broju pronađenih lubanja, u grobnici nalazile 53 žrtve. U slučajevima u kojima je to bilo moguće, kosti su postavljene u anatomski položaj i pregledane kako bi se utvrdila dob, spol, visina i promjene koje su se dogodile

tijekom života ili nakon smrti, te su fotografirane. Sva mjerenja, uz poštivanje etičkih načela, napravljena su u duplikatu. Analize su napravljene na Odjelu za patologiju Opće bolnice u Dubrovniku, Odjelu za sudsku medicinu, Kliničkog zavoda za patologiju, sudsku medicinu i citologiju KBC-a Split, Odjelu za endokrinologiju Klinike za internu medicinu KBC-a Split, te na Nastavnom zavodu za javno zdravstvo Splitsko-dalmatinske županije. Napravljena su detaljna antropometrijska mjerenja i izdvojeni ulomci femura za daljnju analizu. Na izuzetim uzorcima fragmenata obavljeno je niz mjerenja u svrhu određivanja: mase, duljine, promjera s obje strane i specifične težine. Mjerenja su izvršena pomičnom kliznom mjerkom s preciznošću od 0,1 mm i rasponom mjerenja od 0-40 mm, a masa je izmjerena uporabom analitičke vage preciznosti 0,1 mg. i izražena u gramima (g). Svim fragmentima bedrenih kostiju izmjeren je volumen, izražen u centimetrima kubičnim (cm^3), uporabom laboratorijske menzure s rasponom mjerenja do 250 ml. Potom su izračunate vrijednosti prosječne gustoće, izražene u gramima po centimetru kubičnom (g/cm^3) na temelju izmjerenih vrijednosti mase i volumena. DNA profili koštanih ostataka analizirani su i uspoređeni s DNA profilima potencijalnih živih rođaka. DNA profili umnoženi su uporabom AmpFiSTR Yfiler, MiniFiler i Identifiler PCR amplifikacijskog kita (Applied Biosystems, Foster City, CA, USA) u PCR instrumentu ABI Prism 9700. Razdvajanje umnoženih fragmenata napravljeno je upotrebom instrumenta ABI Prism 310 Genetic Analyzer (Applied Biosystems) i GeneMapper ID softvera, verzija 3.2 (Applied Biosystems). Svim fragmentima bedrenih kostiju kvantitativnom metodom - denzitometrijom, izmjerena je površinska gustoća kosti (engl. bone mineral density - BMD) izražena u gramima po centimetru kvadratnom (g/cm^2). U tu je svrhu korišten uređaj denzitometar model QDR 4500 C (S/N 48034). Nakon denzitometrije, na istim uzorcima je izvedena kemijska analiza određivanja masenog udjela kalcija i fosfora. Maseni udio kalcija određen je tehnikom plamene atomske apsorpcione spektrometrije (engl. FAAS, Flame Atomic Absorption Spectroscopy). Mjerenja su obavljena na atomskom apsorpcionom spektrofotometru AAS Vario 6, Analytik Jena. Maseni udio fosfora određen je tehnikom UV VIS spektrofotometrije na UV VIS spektrofotometru Perkin Elmer Lambda 25, double beam. Maseni udio kalcija i fosfora na izuzetim uzorcima tla napravljen je na isti način kao i u uzorcima koštanih fragmenata.

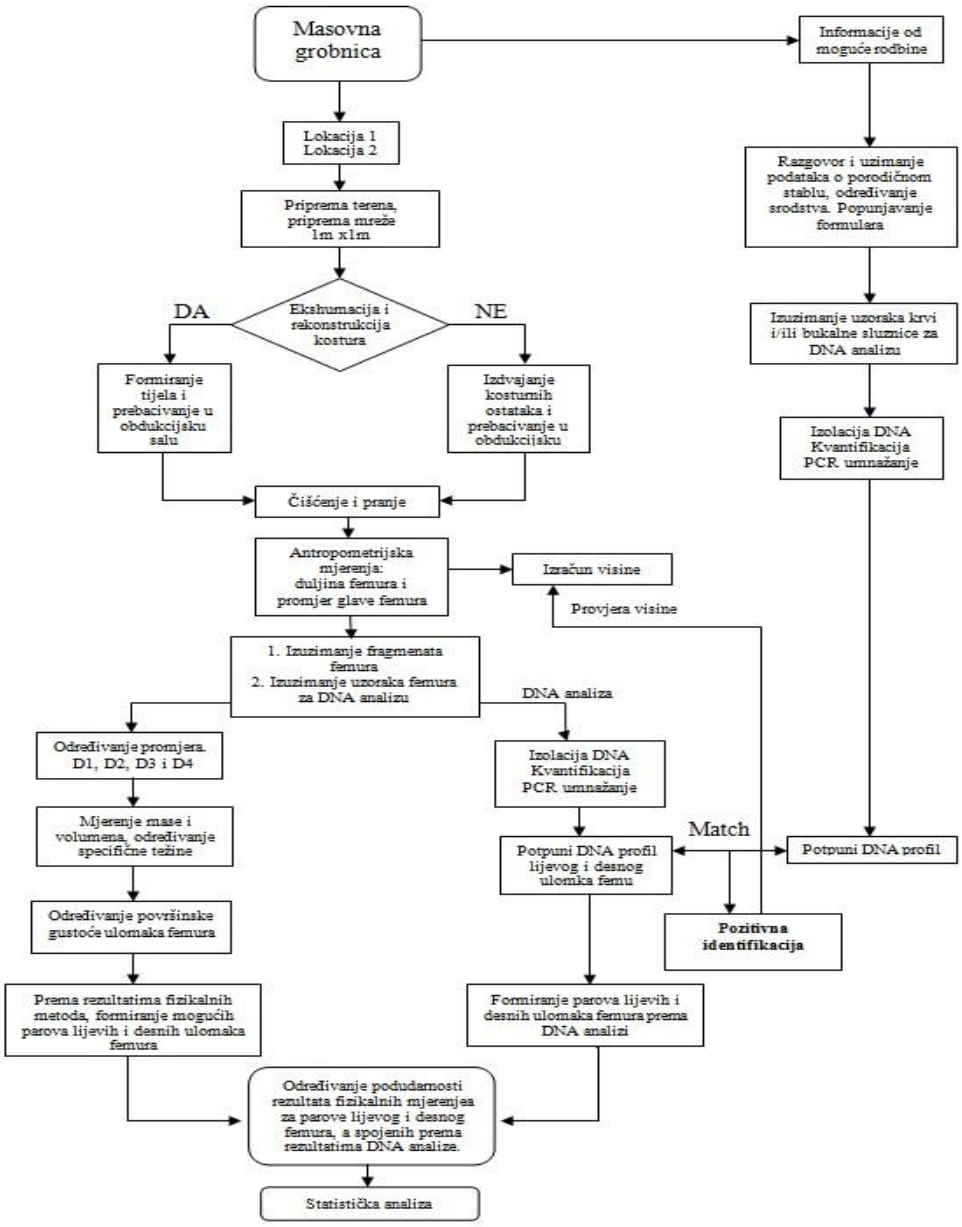
2.2.1. STATISTIČKA OBRADA

Dobiveni rezultati svih mjerenja pohranjeni su u prethodno konstruiranu bazu podataka. Statistička obrada urađena je uporabom softverskih programa GraphPad Prism 4 za Windows, SPSS software 11.03 za Windows i MS Office Excel 2010 paketa. Za sve statističke testove korištena je razina značajnosti od 95% ($P \leq 0,05$).

2.3.REZULTATI

2.3.1. SAŽETAK OBJEDINJENIH REZULTATA

Za potrebe studije, a kako bi se mogli testirati svi predviđeni parametri, odnosno mjerenja, prethodno je konstruiran algoritam - vodič za postupanje po kojim su detaljno razrađene sve faze (14) (Slika 2).



Slika 2. Radni algoritam – prikaz postupaka i fizikalna analiza uzoraka femura.

Detaljna pozornost posvećena je obradi 104 uzorka femura uz pretpostavku da su se u grobnici nalazile, prema broju pronađenih lubanja, 53 žrtve (15). Uzorci femura odabrani su za analizu jer su po svojoj građi čvrste, često dobro uščuvane kosti iz kojih je moguće uspješno izolirati DNK visoke kvalitete. Sparivanjem lijevog i desnog femura iste osobe moguće je prepoloviti broj potencijalnih uzoraka za DNK analizu. Stoga se razmatrala mogućnost kako reducirati broj potrebnih uzroka sparivanjem fragmenata kostiju iste osobe na temelju fizikalnih mjerenja, što je vremenski i financijski značajno, posebice kod obrade masovnih grobnica, koje sadržavaju veliki broj pomiješanih i fragmentiranih kostiju. Antropološka analiza skeletnih ostataka izvršena je na Odjelu za patologiju dubrovačke Opće bolnice.

DNK analiza koštanih ostataka provedena na Odjelu za sudsku medicinu KBC Split. Uporabom DNK analize do danas je uspješno identificirano 18 osoba od 53 nađene (16). Tijekom iskapanja s obje lokacije uzeti su uzorci tla na dvije pozicije - dubine. Zbog nemogućnosti određivanja DNK profila (loša kvaliteta materijala) za sva 104 uzorka bedrenih kostiju, analiza fizikalnih mjerenja napravljena je na 94 uzorka. Od 72 analizirana uzorka, DNK analizom uspješno je formirano 36 parova, na kojima je izmjerena površinska gustoća kostiju, BMD. Izmjerene su i zabilježene duljina femura i promjer glave femura. Potom su izdvojeni dijelovi femura na istim pozicijama. Na izdvojenim fragmentima femura napravljeni su: mjerenje vanjskog promjera krajnjih dijelova koštanih fragmenata, fizikalne veličine; masa, volumen i gustoća, te površinska gustoća (BMD). Nakon mjerenja i izračunavanja svih parametara, uslijedilo je formiranje parova fragmenata femura na temelju svih vrijednosti zabilježenih u bazi podataka. Rezultati formiranja parova fizikalnom analizom uspoređeni su s rezultatima formiranja parova na temelju prethodno obavljene DNK analize. Za sve uspješno formirane parove koštanih fragmenata statističkim izračunom izraženo je odstupanje od podudarnosti u vrijednostima za mjerene parametre između koštanih fragmenata koji čine jedan par, kao mjerilo uspjeha fizikalnih analiza u svrhu formiranja parova. Rezultati sparivanja čitavih bedrenih kostiju iste osobe na temelju duljine i promjera glave femura pokazali su da isto može biti urađeno uz veliku vjerojatnost. Međutim, simulacija sparivanja bedrenih kostiju na temelju fizikalnih mjerenja fragmenata uzetih s istih područja kosti, nije dala zadovoljavajući rezultat. Rezultati dobiveni mjerenjem promjera fragmenata lijeve i desne bedrene kosti na dvije visine i dvije širine nisu pouzdani za

formiranje parova. Specifična težina pokazuje najmanju korelaciju od svih ispitivanih parametara. Površinska gustoća (BMD) testiranih fragmenata femura značajno se razlikovala za lijevi i desni fragment iste osobe (14). Ponekad se umjesto odgovora dobiju nova pitanja, te se krenulo tim putem. Treba istražiti i testirati sve mogućnosti koje bi mogle dati korisne podatke. Kako su rezultati mjerenja gustoće uzoraka ekshumiranih kostiju pokazali stanovite razlike mineralne gustoće lijevog i desnog femura iste osobe, pokušalo se usporediti rezultate dobivene primjenom denzitometrije (DXA) s rezultatima kemijskog određivanja sadržaja kalcija i fosfora u koštanim ostatcima. Kako nije moguće kod živih osoba izvršiti određivanje koncentracije kalcija i fosfora u uzorcima kostiju i usporediti ih s rezultatima mjerenja mineralne gustoće, odlučilo se iskoristiti ovo istraživanje i u te svrhu.

Nakon denzitometrijskog mjerenja mineralne gustoće kosti provedena je kemijska analiza radi određivanja sadržaja kalcija i fosfora u istim uzorcima kostiju i uspoređeni su rezultati. Prema kriterijima sukladnosti rezultata dobivenih denzitometrijom, kemijski je analizirano 20 parova (lijevih i desnih ulomaka iste osobe) podijeljenih u dvije skupine: fragmenti s najmanjim BMD razlikama (10 podudaranja parova, skupina 1) i ulomci s najvećim BMD razlikama (10 podudaranja parova, skupina 2). Analizirani su i uzorci tla, jer tumačenje koncentracija kemijskih elementa u uzorcima kosti mora biti provedeno uzimajući u obzir rezultate analize tla. Naime, tijekom boravka u tlu koštani ostatci podliježu procesu dijageneze, ovisno o okolišnim uvjetima (17,18). Ispitivani uzorci tla bili su neutralni do lagano kiseli. Vrijednosti kalcija varirale su između 23,87 i 31,97%, dok su vrijednosti fosfora bile u rasponu od 9,04 do 13,43%. Odstupanje koncentracije fosfora u uparenim fragmenata lijeve i desne bedrene kosti bilo je manje od odstupanja koncentracije kalcija u istim uzorcima. Rezultati su uspoređeni s obzirom na koncentracije kalcija i fosfora u uzorcima tla, kao i pH vrijednosti. Vrijednosti fosfora u uzorcima kostiju ne razlikuju se od rezultata objavljenih u ranijim studijama (19-21), dok su vrijednosti kalcija bile znatno veće od očekivanih. Korelacija između mineralne gustoće kosti (BMD) i sadržaja kalcija nije dokazana u ispitivanim skupinama, a postoji samo slaba korelacija između BMD i sadržaja fosfora u skupini 1. Rezultati dobiveni u ovom istraživanju ne podržavaju hipotezu da BMD mjeren denzitometrijom ima pozitivnu korelaciju s kemijski određenim

koncentracijama kalcija i fosfora u kostima, što je posebice izraženo za kalcij. Povećane vrijednosti kalcija mogu se objasniti dijagenezom, jer je tlo u području ekshumacije bogato kalcijem, a kalcij biološkog porijekla ne može se razlikovati od kalcija sadržanog u tlu. Stoga, rezultati dobiveni ovom studijom pokazuju da tumačenje rezultata dobivenih samo primjenom denzitometrije kod analize kosturnih ostataka, može voditi pogrešnim zaključcima, jer bez vrijednosti Ca/ P odnosa nije jasno što se zapravo mjeri. Naime, denzitometrija ne može razlikovati sadržaj kalcija (ili fosfora) ugrađen tijekom životnog vijeka od sadržaja kalcija nastalog dijagenezom.

2.3.2. Rezultati rada „Identification of the World War II mass grave victims from the island of Daksa near Dubrovnik, Croatia“

Skeletni ostatci najmanje 53 osobe pronađeni su na dva mjesta označena kao lokacija 1 i Lokacija 2. Radi se o ljudskim kostima starim više od 50 godina, zakopanim u vrijeme koje odgovara Drugom svjetskom ratu. Područje iskapanja označeno je mrežom dimenzija 1x1 metar (Slika 3,4). Kosti su pažljivo iskopane, te označene lokacijom i orijentacijom u odnosu na formiranu mrežu.



Slika 3. Terenski rad, formiranje mreže.



Slika 4. Terenski rad, lubanja *in situ.*

Tamo gdje je bilo moguće, kosti su stavljene u anatomski položaj. Tabelarni i numerički prikaz kostiju napravljen je s obzirom na lokaciju pronalaska i prikazan u tablicama (Tablica 1, 2).

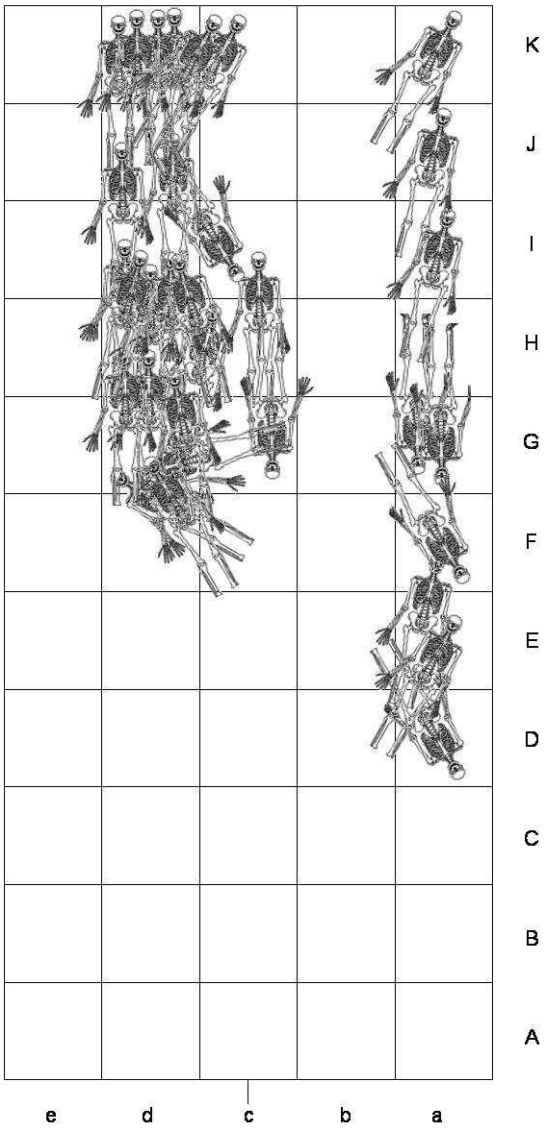
Tablica 1. Prikaz nalaza kostiju, Lokacija 1.

Polje	lopatica	Ključna kost	Nadlaktična kost	Lakatna kost	Palčana kost	Rebra	Prsna kost	Kralježak	Bedrena kost	Zdjelica 1/2	Križna kost	Goljenična kost	Lisna kost	Male kosti	Lubanja
Da	2	2	2	2	2	20	1	20	1	1	0	3	4	82	1
Ea	3	3	3	4	3	29	2	36	5	5	3	3	3	155	1
Fa	3	3	3	2	3	33	1	32	0	1	1	0	0	80	2
Ga	0	0	0	0	0	1	0	4	2	0	0	4	4	97	2
Ha	5	6	6	6	6	84	3	59	6	6	3	2	2	176	0
Ia	0	0	0	0	0	0	0	6	2	0	0	3	4	68	1
Ja	2	2	2	1	1	19	1	28	1	4	2	0	0	63	1
Ka	2	2	2	2	2	17	1	19	0	0	0	0	0	26	1
Ib	0	0	0	0	0	0	0	0	0	0	0	2	1	15	0
Jb	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0
UKUPNO	***17***	***18***	***18***	***18***	***18***	***203***	***9***	***204***	***18***	***17***	***9***	***18***	***18***	***762***	***9***
Fc	0	0	0	0	0	7	0	7	4	3	1	5	6	0	0
Gc	4	4	3	1	1	31	1	23	1	3	2	1	1	81	1
Hc	7	4	6	6	6	52	5	63	10	6	6	3	3	200	1
Ic	3	3	5	3	4	33	2	33	1	4	2	0	0	93	2
Kc	5	4	5	6	5	75	2	58	0	3	3	0	0	188	2
Fd	0	0	0	0	0	0	0	1	4	1	1	3	3	13	0
Gd	4	4	6	7	6	81	4	58	8	7	1	14	14	281	3
Hd	4	5	3	3	5	59	2	69	3	4	2	4	4	175	3
Id	7	7	7	9	8	60	2	71	3	3	1	12	13	428	5
Jd	4	4	3	2	3	68	2	51	12	6	4	4	2	86	2
Kd	6	7	8	6	8	62	3	65	0	3	0	0	0	173	4
UKUPNO	***44***	***42***	***46***	***43***	***46***	***528***	***23***	***499***	***46***	***43***	***23***	***46***	***46***	***1718***	***23***
SVEUKUPNO	**61**	**60**	**64**	**61**	**64**	**731**	**32**	**703**	**64**	**60**	**32**	**64**	**64**	**2480**	**32**

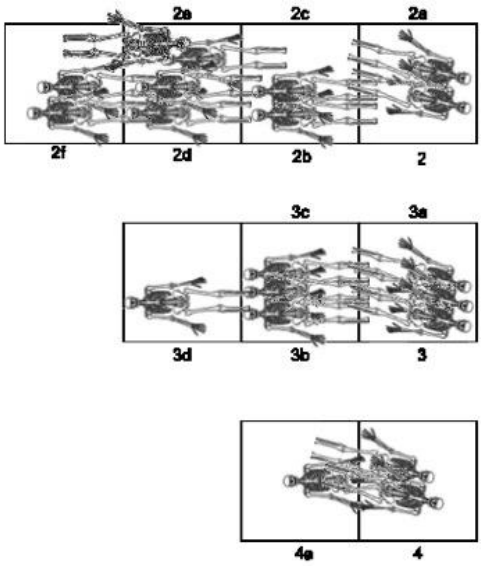
Tablica 2. Prikaz nalaza kostiju, Lokacija 2.

Polje	Lopatica	Ključna kost	Nadlaktična kost	Lakatna kost	Palčana kost	Rebra	Prsna kost	Kralježak	Bedrena kost	Zdjelica 1/2	Križna kost	Goljenična kost	Lisna kost	Male kosti	Lubanja
2	2	3	4	1	3	23	1	31	3	3	2	2	2	25	2
2a	2	3	3	4	4	35	2	43	5	5	2	5	4	75	0
2b	2	2	1	2	2	27	1	23	0	0	0	4	6	231	2
2c	2	0	2	1	1	4	0	13	4	10	5	7	6	28	0
2d	3	3	3	3	3	57	2	55	6	1	0	0	0	35	2
2e	6	5	3	3	3	37	3	35	0	0	1	0	0	76	2
2f	1	3	3	4	4	27	1	34	2	1	0	2	2	89	2
Ukupno	**18**	**19**	**19**	**18**	**20**	**210**	**10**	**234**	**20**	**20**	**10**	**20**	**20**	**559**	**10**
3	2	3	2	2	2	14	0	21	2	2	1	6	8	181	3
3a	4	3	3	4	3	37	2	40	7	2	1	6	4	69	0
3b	2	1	3	3	4	36	2	28	5	10	4	1	1	115	1
3c	6	7	6	3	3	68	3	66	0	0	1	1	0	57	2
3d	0	0	0	2	2	4	0	9	0	0	0	0	1	17	1
Ukupno	**14**	**14**	**14**	**14**	**14**	**159**	**7**	**164**	**14**	**14**	**7**	**14**	**14**	**439**	**7**
4	6	6	5	3	4	69	3	36	4	5	2	2	1	67	2
4a	0	0	0	1	2	0	0	6	2	0	0	4	5	84	1
Ukupno	**6**	**6**	**5**	**4**	**6**	**69**	**3**	**42**	**6**	**5**	**2**	**6**	**6**	**151**	**3**
5	0	0	1	1	0	0	0	0	0	0	0	1	0	3	1
Sveukupno	**38**	**39**	**39**	**37**	**40**	**438**	**20**	**440**	**40**	**39**	**19**	**41**	**40**	**1152**	**21**

S obzirom na mjesto otkrivanja pojedinih kostiju i njihovu orijentaciju unutar mreže, napravljen je shematski prikaz položaja određenog tijela u odnosu prema drugim tijelima i njihovoj prostornoj orijentaciji unutar označenih shematskih stranica (Shema 1, Shema 2).



Shema 1. Shematski prikaz nalaza, Lokacija 1.

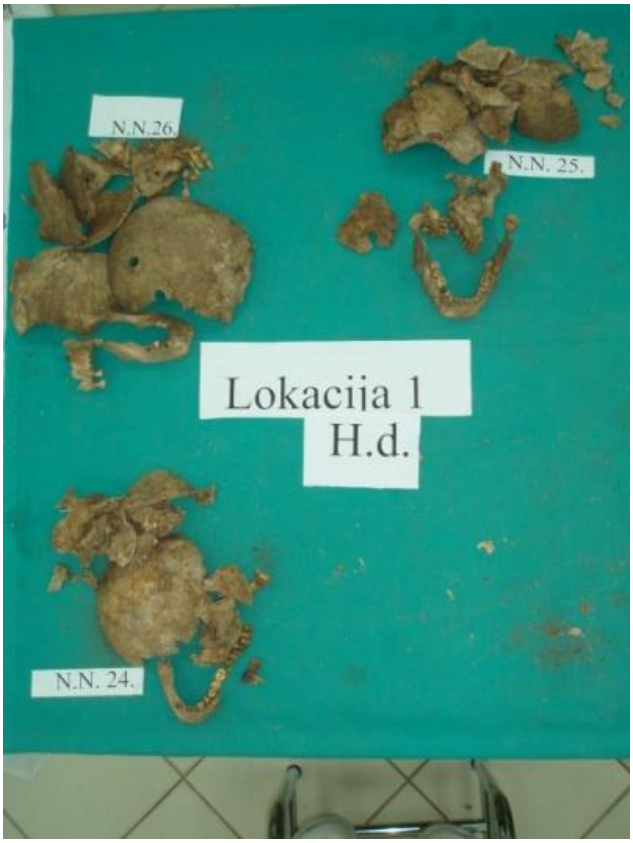


Shema 2. Shematski prikaz nalaza, Lokacija 2.

Skeletni ostaci imaju karakterističan muške antropomorfne karakteristike. Strijelne rane su identificirane u 22 osobe. Praktički sve uočene ulazne strijelne rane lokalizirane su u zatiljnom području (Slika 5, 6, 7).



Slika 5. Rad u sali, nalaz u kvadrantu H.d. Lokacija 1.



Slika 6. Rad u Sali, nalaz lubanja u kvadrantu H.d. Lokacija 1.



Slika 7. Pronađeni predmeti Lokacija 1.

DNA je uspješno izolirana iz 49 od ukupno 53 posmrtnih ostataka. Korištenjem AmpFlSTR Yfiler PCR amplifikaciju Kit, dobiveno je 34 od 49 profila, od toga 32 puna profila i 2 parcijalna profila. Korištenjem MiniFiler Amplificaton Kit dobiveno je 40 od 49 DNK profila, uz samo dva parcijalna. Identifiler PCR Amplificaton Kit korišten je u 4 slučaja, a dobiveno je 2 puna profila, kao i 2 parcijalna profila. DNK je izolirana iz uzoraka krvi 23 živih srodnika. Unakrsno podudaranje rezultata s podatcima srodnika rezultiralo je s 18 pozitivnih identifikacija.

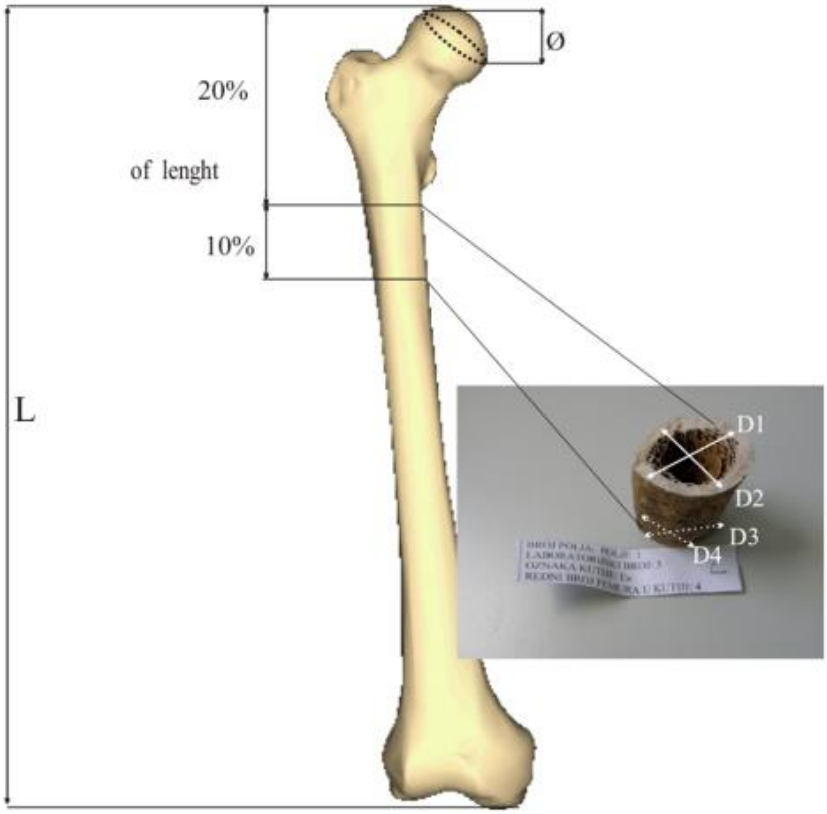
2.3.3. Rezultati rada „Identification process of skeletal remains from mass graves: our experience and proposal guidelines“

Istraživanje je obuhvatilo analizu bedrenih kostiju, ekshumiranih na dvije mikrolokacije (Lokacija 1 i Lokacija 2) na otočiću Daksi kraj Dubrovnika. Pojedinačno je izmjerena i zabilježena duljina i promjer glave svakog femura (Tablica 3).

Tablica 3.Sparivanje bedrenih kostiju (Lokacija 1) iste osobe na temelju lokacije pronalaska, duljine i promjera glave bedrene kosti. Urađen je i izračun visine, te određen spol.

Oznaka	Broj	Duljina	Strana	Promjer glave	Visina osobe	Spol
Da	1	40,8	L	4,8	158cm Coxa vara	muški
Ea	1	40,7	D	5,1	Nepouzdano	
	2	43,9	L	4,6	166,4 ±3,27 cm	muški
	3	44,1	D	4,7		
	4	46,3	L	5,1	172 ±3,27 cm	Muški
	5	46,5	D	5		
Ga	1	46	L	5,2	170 ±3,27 cm	Muški
	2	45,5	D	5,2		
Ha	1	51,5	L	5,1	184 ±3,27 cm	Muški
	2	51,5	D	5,1		
	3	46,5	L	4,9	172 ±3,27 cm	Muški
	4	46,4	D	4,9		
	5	50	L	5,0	180 ±3,27 cm	Muški
	6	50	D	5,0		
Ia	1	47	L	5,0	173 ±3,27 cm	Muški
	2	46,7	D	5,0		
Ja	1	47,4	D	4,9	174 ±3,27 cm	Muški
Jb	1	ošt	L	4,9		
Fc	1	43	L	4,6	165 ±3,27 cm	Muški
	2	43,5	D	4,7		
	3	ošt	L	ošt	Nepoznato	Nepoznato
	4	ošt	D	ošt	Nepoznato	Nepoznato
Gc	1	ošt	L	5,5	Nepoznato	Muški
Hc	1	49	D	5,0	178 ±3,27 cm	Muški
	2	50	D	5,1	180 ±3,27 cm	Muški
	3	46,1	L	5,0	171 ±3,27 cm	Muški
	4	43,8	L	4,7	165,5 ±3,27 cm	Vjerojatno muški
	5	ošt	D	ošt	nepoznato	Nepoznato
	6	ošt	D	4,7	Nepoznato	Vjerojatno muški
	7	ošt	L	4,7		
	8	ošt	D	5,0	Nepoznato	Muški
	9	ošt	D	5,0	Nepoznato	Muški
	10	ošt	L	5,1	Nepoznato	Muški
Ic	1	ošt	L	ošt	Nepoznato	Nepoznato
Fd	1	52	L	5,2	185 ±3,27 cm	Muški
	2	41,5	L	4,7	160 ±3,27 cm	Vjerojatno muški
	3	ošt	D	4,9	Nepoznato	Muški
	4	ošt	D	ošt	Nepoznato	Nepoznato
Gd	1	47,2	L	5,3	174 ±3,27 cm	Muški
	2	47,1	D	5,3		
	3	45,9	L	4,9	172 ±3,27 cm	Muški
	4	46,4	D	4,9		
	5	ošt	L	5,0	Nepoznato	Muški
	6	ošt	D	5,2	Nepoznato	Muški
	7	ošt	D	ošt	Nepoznato	Nepoznato
	8	ošt	L	ošt	Nepoznato	Nepoznato
Hd	1	41,7	L	4,7	161 ±3,27 cm	Vjerojatno muški
	2	42	D	4,7		
	3	ošt	L	4,7	168,8 ±3,27 cm	Muški
Id	1	45,1	D	4,9		
	2	40,5	L	4,2	157,8 ±3,27 cm	Moguće muški
	3	ošt	D	4,2		
Jd	1	48	L	4,8	176,8 ±3,27 cm	Muški
	2	48,5	D	4,8		
	3	48	L	5,3	175,6 ±3,27 cm	Muški
	4	48	D	5,3		
	5	45	L	4,8	168,5 ±3,27 cm	Muški
	6	45	D	4,9		
	7	46,5	L	5,1	172,8 ±3,27 cm	Muški
	8	46,8	D	5,1		
	9	44,6	L	5,0	167,8 ±3,27 cm	Muški
	10	44,7	D	4,9		
	11	47,5	L	5,0	174,5 ±3,27 cm	Muški
	12	ošt	D	5,1		

Potom su izdvojeni dijelovi femura na istim pozicijama (Slika 8).



Slika 8. Pozicija mjerenja dužine femura (L), promjera glave femura (Ø) i promjeri koštanih fragmenata, gornji širi promjer (D1), gornji uži promjer (D2), donji širi promjer (D3) i donji uži promjer (D4).

Na izdvojenim fragmentima femura napravljeni su: mjerenje vanjskih promjera krajnjih dijelova koštanih fragmenata, mjerenje mase i volumena.

Na temelju izmjerenih vrijednosti mase i volumena izračunate su vrijednosti prosječne gustoće.

Zbog nemogućnosti određivanja DNK profila (loša kvaliteta materijala) za sva 104 uzorka bedrenih kostiju, analiza fizikalnih mjerenja napravljena je na 94 uzorka. Od 72 analizirana uzorka, DNK analizom uspješno je formirano 36 parova, na kojima je izmjerena površinska gustoća kostiju, BMD. Nakon mjerenja i izračunavanja svih parametara, uslijedilo je formiranje parova fragmenata femura na temelju svih vrijednosti zabilježenih u bazi podataka. Rezultati formiranja parova fizikalnom analizom uspoređeni su s rezultatima formiranja parova, na temelju prethodno obavljene DNK analize. Za sve uspješno formirane parove koštanih fragmenata, statističkim izračunom izraženo je odstupanje od podudarnosti u vrijednostima za

mjerene parametre između koštanih fragmenata koji čine jedan par, kao mjerilo uspjeha fizikalnih analiza u svrhu formiranja parova (Tablica 4).

Tablica 4. Razlike i podudarnosti (ako je razlika = 0%) između izmjerenih vrijednosti lijevih i desnih femura (29 parova): duljina femura (L); promjer glave femura (Ø); promjer gornjeg, šireg dijela fragmenta kosti (D1); promjer gornjeg, užeg dijela fragmenta kosti (D2); promjer donjeg, šireg dijela fragmenta kosti (D3); promjer donjeg, užeg dijela fragmenta kosti (D4) (vidi Sliku 8); prosječna gustoća (ρ) vrijednost površinske gustoće kosti (BMD)

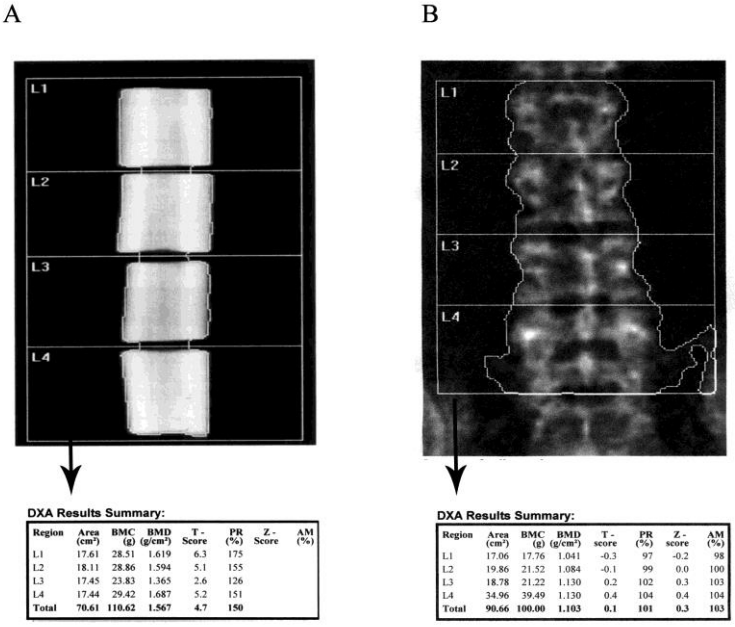
Par	Razlike između izmjerenih vrijednosti lijevog i desnog femura: Odvojeno za svaki par (%)							
	L	Ø	D1	D2	D3	D4	ρ	BMD
1	0.25*	-6.25ᵀ	3.39*	1.46*	0.28*	4.86*	1.28*	-0.15*
2	-0.46*	-2.17*	4.93*	-3.92*	-4.12*	-0.79*	-3.51*	-1.94*
3	1.73*	1.96*	2.62*	15.02ǂ	-5.06ᵀ	12.67ᵀ	7.59ᵀ	3.97*
4	1.09*	0.00	0.59*	-7.17ᵀ	-5.23ᵀ	-5.37*	12.71ᵀ	0.95*
5	0.00	0.00	0.00	1.38*	2.22*	4.21*	-11.41ᵀ	14.42ᵀ
6	0.22*	0.00	2.81*	-3.64*	-3.16*	1.43*	29.96ǂ	2.73*
7	0.00	0.00	-0.62*	1.57*	1.59*	-4.38*	0.00	13.24ᵀ
8	0.64*	0.00	-0.32*	3.45*	-0.65*	2.27*	-5.00ᵀ	7.50ᵀ
9	-3.27*	0.00	-2.87*	1.83*	3.63*	7.53ᵀ	-13.42ᵀ	-5.06ᵀ
10	-1.16*	-2.17*	-2.53*	4.00*	-5.59ᵀ	3.56*	-6.13ᵀ	4.80*
11	-0.72*	2.13*	5.26ᵀ	1.40*	1.72*	1.90*	-2.52*	2.07*
12	0.25*	0.00	-1.10*	1.13*	0.76*	0.00	-76.09ᵀ	0.77*
13	-1.04*	0.00	-0.32*	5.56ᵀ	-7.30ᵀ	-2.33*	-0.56*	-4.41*
14	0.00	0.00	5.99ᵀ	2.55*	8.94ᵀ	2.59*	3.35*	-0.93*
15	0.00	-2.08*	-10.14ᵀ	-1.52*	-9.09ᵀ	3.80*	4.85*	-4.55*
16	-0.65*	0.00	7.14ᵀ	-4.68*	9.76ᵀ	0.38*	8.18ᵀ	9.30ᵀ
17	-0.22*	2.00*	5.03ᵀ	6.29ᵀ	0.36*	6.59ᵀ	-25.32ǂ	3.36*
18	0.21*	-2.00*	3.51*	-0.73*	-1.02*	0.36*	15.20ǂ	-1.56*
19	0.00	-4.26*	5.96ᵀ	8.80ᵀ	10.14ᵀ	0.00	1.01*	-0.58*
20	-0.42*	1.92*	-3.19*	0.66*	0.97*	1.05*	5.57ᵀ	5.97ᵀ
21	0.23*	-2.04*	11.55ᵀ	-8.07ᵀ	-2.62*	0.00	6.32ᵀ	1.62*
22	0.00	1.89*	2.70*	-3.19*	-0.31*	-1.12*	36.26ǂ	2.13*
23	0.00	0.00	3.72*	4.81*	9.41ᵀ	2.43*	-0.59*	-5.90ᵀ
24	-1.11*	0.00	-0.72*	-1.67*	-0.74*	-0.42*	-5.05ᵀ	4.04*
25	-1.27*	0.00	-6.87ᵀ	5.63ᵀ	-9.12ᵀ	3.04*	-19.38ǂ	1.64*
26	1.79*	-3.77*	1.50*	4.98*	2.56*	1.95*	20.22ǂ	-0.53*
27	0.22*	0.00	0.00	0.68*	0.93*	1.37*	16.30ǂ	-3.29*
28	-0.23*	0.00	0.00	0.40*	3.38*	-6.56ᵀ	6.74ᵀ	-4.56*
29	1.74*	-4.35*	0.00	-1.54*	2.08*	3.35*	13.50ᵀ	1.54*

Razlike i podudarnosti	Razlike izmjerenih vrijednosti 29 femoralni parova (%)							
	L	Ø	D1	D2	D3	D4	ρ	BMD
Podudarnost 100%	25.9	51.7	13.8	0	6.9	0	3.4	0
* Razlika ≤ 5%	74.1	44.8	58.6	65.5	72.4	69.0	27.6	51.7
ᵀ Razlika od 5-15%	0	3.4	27.6	34.5	20.7	27.6	37.9	37.9
ǂ Razlika ≥ 15%	0	0	0	0	0	3.4	31.0	10.3
§Rezultati *T testa* primjenjenih na uzorke lijevog i desnog femura, odnosno femoralnih fragmenata (N= 29 parova)	0.984	0.946	0.868	0.747	0.758	0.800	0.675	0.829

§T-test je značajan na razini 0.01 uz pouzdanost 95%

2.3.4. Rezultati rada „Bone mineral density of skeletal remains: Discordant results between chemical analysis and DXA method“

Fragmenti femura su zbog svog specifičnog izgleda i veličine poredani u niz od četiri, kako bi se analizirali programom za lumbalnu kralježnicu. Uzorci su skenirani normativnim programom muškaraca bijele rase (težine 75 kg, visina 175 cm). DXA rezultati pokazali su razlike u vrijednosti BMD između fragmenata lijeve i desne bedrene kosti iste osobe (Slika 9).



Region	Area (cm²)	BMC (g)	BMD (g/cm²)	T-Score	PR (%)	Z-Score	AM (%)
L1	17.61	28.51	1.619	6.3	175		
L2	18.11	28.86	1.594	5.1	155		
L3	17.45	23.83	1.365	2.6	126		
L4	17.44	29.42	1.687	5.2	151		
Total	**70.61**	**110.62**	**1.567**	**4.7**	**150**		

Region	Area (cm²)	BMC (g)	BMD (g/cm²)	T-score	PR (%)	Z-score	AM (%)
L1	17.06	17.76	1.041	-0.3	97	-0.2	98
L2	19.86	21.52	1.084	-0.1	99	0.0	100
L3	18.78	21.22	1.130	0.2	102	0.3	103
L4	34.96	39.49	1.130	0.4	104	0.4	104
Total	**90.66**	**100.00**	**1.103**	**0.1**	**101**	**0.3**	**103**

Slika 9. Denzitometrija femoralnih fragmenata. Niz od četiri femoralna fragmenta (A) poredanih da oponašaju slabinsku kralježnicu (B) radi upotrebe programa za analizu slabinske kralježnice

Nakon mjerenja mineralne gustoće kosti (BMD) denzitometrom, provedena je kemijska analiza radi kvantificiranja sadržaja kalcija i fosfora u uzorcima kostiju i njihovih međusobnih omjera. Prema kriterijima sukladnosti rezultata dobivenih denzitometrijom, kemijski je analizirano 20 parova (lijevih i desnih ulomaka iste osobe) podijeljenih u dvije skupine: fragmenti s najmanjim BMD razlikama (10 podudaranja parova, skupina 1) i ulomci s najvećim BMD razlikama (10 podudaranja parova, skupina 2). Utjecaj dijageneze na kalcij i fosfor u uzorcima kostiju utvrđen je određivanjem sadržaja kalcija i fosfora, te pH vrijednosti u

uzorcima tla uzetim tijekom iskopavanja. Uzeta su po dva uzorka za svaku lokaciju: prvi uzorak je iz razine skeletnih ostataka, a drugi 20 cm ispod njih. Uzorci su pripravljeni i analizirani na isti način kao i uzorci kostiju. Ispitivani uzorci tla su bili neutralni do lagano kiseli. Vrijednosti kalcija variraju između 23,87 i 31,97%, dok su vrijednosti fosfora u rasponu od 9,04 do 13,43%. Odstupanje koncentracije fosfora u uparenim fragmenata lijeve i desne bedrene kosti bilo je manje od odstupanja koncentracije kalcija u istim uzorcima. Rezultati su uspoređeni s obzirom na koncentracije kalcija i fosfora u uzorcima tla, kao i pH vrijednosti. Lokacija oznake 1 imala je manji pH tla, što objašnjava nešto veće vrijednosti kalcija u kostima, jer je kiseli okoliš povoljniji za bolje otapanje kalcija. Tlo Lokacije 2 je blago alkalno, što je rezultiralo manjim vrijednostima kalcija u uzorcima kosti u usporedbi s uzorcima kosti s Lokacije 1 (Tablica 5).

Tablica 5.DXA i kemijska analiza skeletnih ostataka (BMD, BMD devijacija, sadržaj Ca i P, i odnos Ca/P).

		Skupina 1*					Skupina 2**				
Uzorak No.	Fragment femura	BMD g/cm2	BMD Devijacija* (%)	% Ca	% P	Ca/P odnos	BMD g/cm2	BMD Devijacija* (%)	% Ca	% P	Ca/P odnos
1	L	1.316	0.15	29.12	10.22	2.849	1.409	5.04	26.47	10.71	2.472
	R	1.314		27.26	10.27	2.654	1.480		27.42	10.51	2.609
2	L	1.521	0.53	30.45	11.31	2.692	1.412	5.06	28.43	11.56	2.459
	R	1.513		29.47	10.8	2.729	1.344		29.17	11.58	2.518
3	L	1.563	0.58	26.01	10.08	2.580	1.776	5.9	25.75	9.88	2.606
	R	1.554		24.6	9.04	2.721	1.677		23.87	10.05	2.374
4	L	1.297	0.77	28.09	10.36	2.711	1.382	5.93	24.13	11.57	2.086
	R	1.307		29.58	10.53	2.809	1.464		25.77	10.5	2.454
5	L	1.741	0.93	27.41	12.24	2.239	1.575	6.35	27.82	11.82	2.354
	R	1.725		25.31	11.9	2.127	1.675		25.6	11.3	2.265
6	L	1.456	0.96	26.48	11.58	2.287	1.394	8.11	28.83	13.43	2.146
	R	1.470		29.96	11.47	2.612	1.507		27.51	11.41	2.411
7	L	1.439	1.05	30.77	12.06	2.551	1.248	10.26	27.98	11.7	2.392
	R	1.424		26.37	9.71	2.716	1.376		31.46	11.5	2.737
8	L	1.299	1.56	30.12	10.63	2.833	1.387	11.41	25.5	10.09	2.528
	R	1.279		31.97	12.62	2.533	1.254		25.43	9.68	2.627
9	L	1.594	1.57	27.59	10.68	2.593	1.396	15.26	29.3	10.8	2.713
	R	1.619		26.09	10.91	2.391	1.609		30.01	11.16	2.688
10	L	1.338	1.64	26.64	9.68	2.751	1.531	16.85	29.68	11.17	2.656
	R	1.360		26.2	9.9	2.646	1.789		30.89	11.35	2.722
Medijan		1.456	0.974	27.975	10.800	2.601	1.484	9.017	27.551	11.089	2.491
Standard. devijacija		0.144	0.497	2.067	0.954	0.200	0.157	4.298	2.185	0.856	0.187
Minimum		1.279	0.15*	24.60	9.04	2.127	1.248	5.04**	23.87	9.68	2.086
Maximum		1.741	1.64*	31.97	12.52	2.849	1.789	16.85**	31.46	13.43	2.737

*Devijacija površinske gustoće između lijevog (L) i desnog (R) fragmenta femura (Skupina 1) je manja ili jednaka 5%
**Devijacija površinske gustoće između lijevog (L) i desnog (R) fragmenta femura (Skupina 2) je veća od 5%

Kiselost tla nije bila u korelaciji s koncentracijom fosfora (Tablica 6).

Tablica 6. Resultati BMD, sadržaja kalcija i fosfora, odnos kalcija-fosfora u kostima, dva uzorka tla s dvije lokacije (prvi uzorak tla uzet je u razini skeletnih ostataka, a drugi uzorak uzet je 20cm ispod skeletnih ostataka).

Parametri		Lokacija 1		Lokacija2	
		Uzorci kosti (N=22)	Uzorci tla (srednja vrijednost)	Uzorci kosti (N=18)	Uzorci tla
BMD (g/cm^2)	Minimum	1.248		1.245	
	Maximum	1.789		1.776	
	Median	1.409		1.517	
Ca (%)	Minimum	25.310		23.87	
	Maximum	31.969		30.77	
	Median	28.975	13.3	26.050	12.5
P (%)	Minimum	10.220		9.040	
	Maximum	13.430		12.060	
	Median	11.380	0.068	10.295	0.073
Ca/P ratio	Minimum	2.127		2.086	
	Maximum	2.849		2.751	
	Median	2.611		2.587	
pH (5% -vodene otopine)			6.8		7.5

U odnosu na BMD i fosfora, utvrđena je slaba korelacija s uzorcima uzetima iz Skupine 1 (r = 0,265, *P* = 0,259). Za uzorke iz Skupine 2 utvrđena je negativna korelacija (r = -0,155, *P* = 0,514). U odnosu BMD i kalcija, za obje skupine ne postoji korelacija. Faktor korelacije za uzorke iz Skupine I je -0,447 (*P* = 0.048), a za uzorke Skupine II faktor je -0,011 (*P* = 0,964) .Utvrđena je značajna parcijalna korelacija između BMD i pridruženih koncentracija kalcija i fosfora u obje testirane skupine (posebice za Skupinu I, r = 0.7365, $P \leq 0,05$) (Tablica 7).

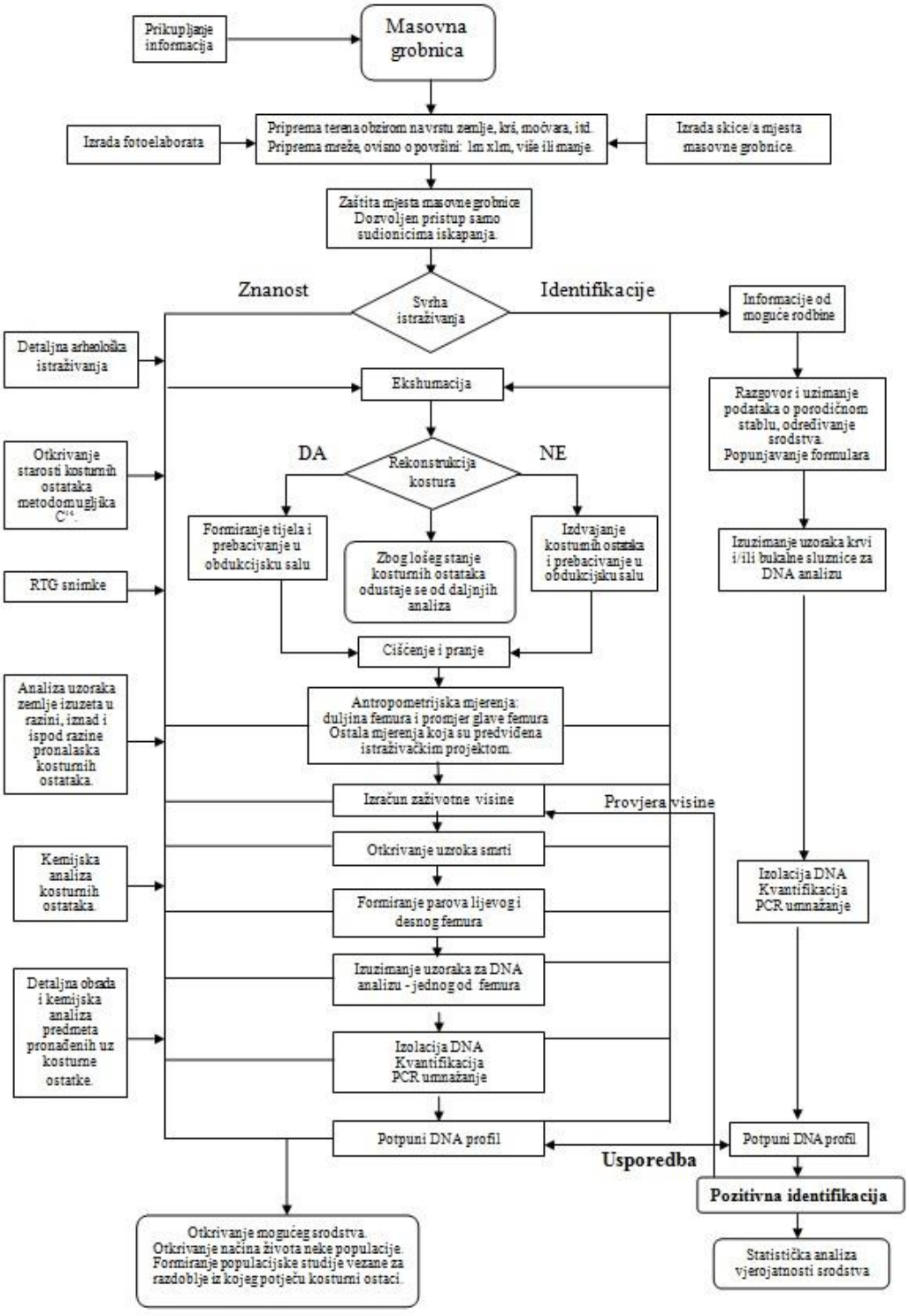
Tablica 7. Koeficijenti korelacije za skupinu I i II (N= 20 u svakoj skupini).

Parovi	Skupina I		Skupina II	
	Koeficijenti korelacije parova (r)	*P*	Koeficijenti korelacije parova (r)	*P*
BMD & P	0.265	0.259	-0.155	0.514
BMD & Ca	-0.447	0.048	-0.011	0.964
	Parcijalni koeficijent korelacije			
	Ca		P	
Ca	1.0000		0.4965† (*P*=0.031)	
P	0.7365* ($P \leq 0.05$)		1.0000	

*jaka korelacija
†značajna korelacija

2.4. RASPRAVA

Glavni je cilj ekshumacije masovnih grobnica odrediti minimalni broj žrtava (ako ne uvijek točan), utvrditi uzrok i način smrti, te po mogućnosti provesti identifikaciju žrtava, radi povratka posmrtnih ostataka obiteljima. Brižljivim pristupom radu na terenu, te kasnijim radom u obdukcijskoj sali, moguće je naknadno rekonstruirati položaj i odnose skeletnih ostataka, kombiniranjem podataka prikupljenih tijekom ekshumacije s podatcima antropometrijskih mjerenja, te time smanjiti broj uzoraka i olakšati identifikaciju. Ukoliko nedovoljno educirane osobe rukovode ekshumacijom, moguće greške često se kasnije ne mogu ispraviti. Predložene smjernice uključuju niz različitih postupaka i / ili analize. Treba primijetiti da slične smjernice nisu pronađene u dostupnoj literaturi, te stoga one mogu biti korisne svima koji počinju rad na ekshumaciji i identifikaciji žrtava masovnih grobnica, kao i onima koji imaju dugogodišnje iskustvo u tom poslu. (Slika 10).



Slika 10. Algoritam predloženih smjernica obrade uzoraka iz masovne grobnice.

Antropometrijski podatci i status zubi imaju izrazito ograničenu ulogu u identifikaciji žrtava masovnih grobnica koje datiraju iz Drugog svjetskog rata, uglavnom zato jer ne postoje ante mortem podatci vezani za žrtve. Treba reći da, uz dobro organizirani rad, žrtve masovne grobnice mogu "ispričati priču" o svojim posljednjim trenutcima, dok je identifikacija žrtava praktički isključivo u domeni DNK analize (3-5), koja je bila imperativ u ovom istraživanju. Zubi imaju prioritet kao izvor uzorka, ali u situacijama u kojima se ne nalaze zubi ili lubanje ili kad postoje razlike između broja lubanja i ostalih kostiju ekshumiranih osoba, femur je logičan izbor kao izvor uzoraka za DNK analizu.

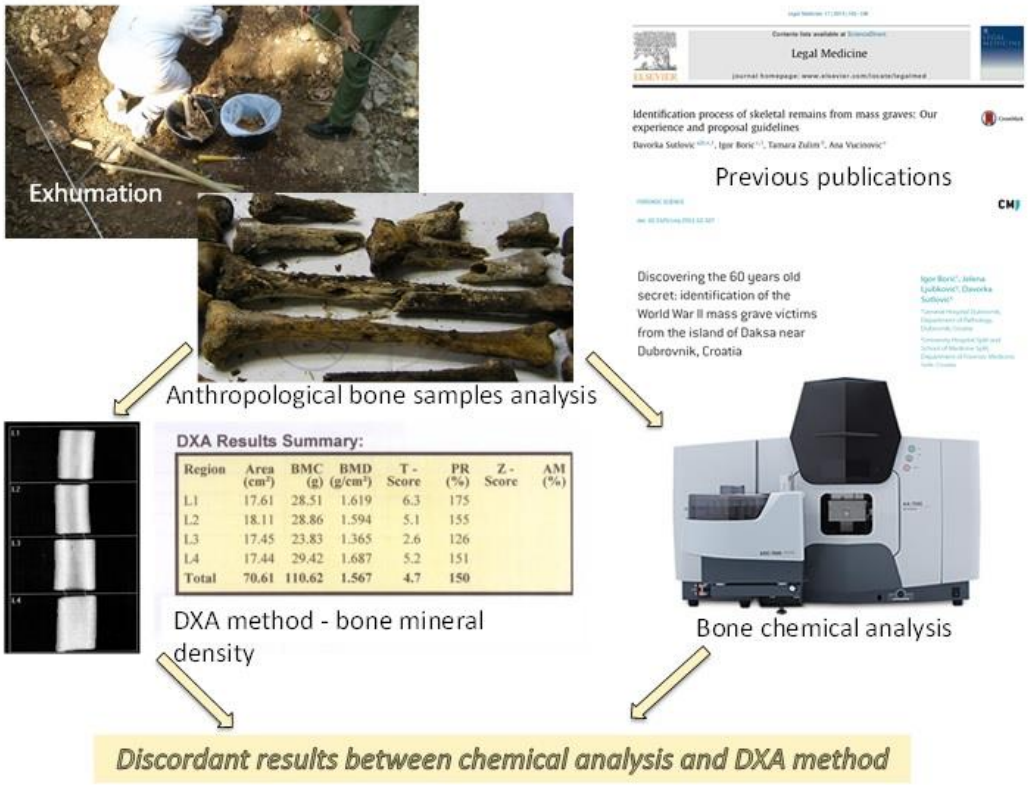
Simulacija sparivanja bedrenih kostiju na temelju fizikalnih mjerenja fragmenata uzetih s istih područja kosti, nije dala zadovoljavajući rezultat:

1. Rezultati dobiveni mjerenjem promjera fragmenata lijeve i desne bedrene kosti na dvije visine i dvije širine nisu pouzdani za formiranje parova.
2. Specifična težina pokazuje najmanju korelaciju od svih ispitivanih parametara.
3. Površinska gustoća (BMD) testiranih fragmenata femura značajno se razlikovala za lijevi i desni fragment iste osobe.

Istraživanje je provedeno kako bi se provjerila podudarnost između DXA utvrđene mineralne gustoće kostiju (BMD) i kemijski analiziranog mineralnog sadržaja arheoloških uzoraka (bedrene kosti). Mineralna gustoća kostiju varira između kostiju, pa čak i između različitih dijelova iste kosti (16). Denzitometrija DXA izračunava iznos količinu hidroksiapatita u kostima kao mineralni sadržaj kosti BMC u gramima, te izračunava BMD izražen u g/cm2. To je uglavnom u skladu s koncentracijama kalcija i fosfora. Odnos Ca / P u hidroksiapatitu in vitro je 1,5-1,67 (22), dok je omjer Ca / P u hidroksiapatitu ljudskih kostiju stalan i iznosi 2,2 (20,23). Do sada nitko nije određivao omjer Ca /P u skeletnim uzorcima. Nekoliko studija određuje omjer Ca / P iz uzoraka kostiju uzetih 24 sata nakon smrti (20,21), a rezultati su bili u skladu s omjerom Ca / P kod žive osobe.

Tumačenje koncentracija mineralnih elementa iz ekshumiranih kostiju mora biti u kontekstu s analizom tla i drugih okolišnih faktora. Tijekom boravka u tlu, kost se podvrgava procesu dijageneze (18,24). Jedna od prvih promjena tijekom koštane dijagenezi je otapanje minerala i njihova razmjena s okolnim tlom (25). Lokacija istraživane masovne grobnice je smještena je na malom otoku ($0.07km^2$) okruženom Jadranskim morem. Otok se sastoji od crvenice, dolomita i vapnenačkih stijena i

minerala (26). Tlo na mjestu ekshumacije je u blizini borove šume i ima neutralne do lagano kisele pH vrijednosti. Vrijednosti fosfora u uzorcima kostiju ne razlikuju se od rezultata objavljenih u ranijim studijama (27-30), dok su vrijednosti kalcija znatno veće nego što se očekivalo. Lokacija 1 ima manji pH tla, što objašnjava nešto veće vrijednosti kalcija u kostima, jer je kiseli okoliš povoljniji za bolje otapanje kalcija. Tlo Lokacije 2 blago je alkalno, što je rezultiralo manjim vrijednostima kalcija u uzorcima kosti u usporedbi s uzorcima kosti s Lokacije 1. Omjer kalcija i fosfora bio je stoga veći, što ukazuje na utjecaj karakteristika tla na dijagenezu kosti. Korelacija između BMD i količine kalcija nije dokazana u ispitivanim skupinama, a samo slaba korelacija između BMD i količine fosfora identificirana je samo u Skupini 1. Rezultati dobiveni u ovom istraživanju ne podržavaju hipotezu da će BMD mjerena denzitometrijom imati pozitivnu korelaciju s kemijski određenim koncentracija kalcija i fosfora u kostima, što je posebno izraženo za kalcij. Budući da je tlo u području istraživane lokacije bogato kalcijem, a kalcij biološkog podrijetla ne može se razlikovati od kalcija koji se nalazi u tlu, povećane vrijednosti kalcija u kostima mogu se objasniti dijagenezom (Slika 11).



Slika 11. Nepodudarni rezultat između kemijske analize i denzitometrije.

Rezultati dobiveni ovim istraživanjem pokazuju da tumačenje rezultata napravljenih na uzorcima iz skeletnih ostataka, a dobivenih samo primjenom metode denzitometrije, može biti pogrešno. Naime, bez određivanja vrijednosti omjera Ca / P u uzorcima kosturnih ostataka i njihovim koncentracijama u uzorcima zemlje iz okoliša, ne postoji sigurnost u interpretaciju rezultata. Teško je razlučiti radi li se o stvarnim zaživotnim koncentracijama, a time i meneralnoj gustoći kostiju ili pak posljedici dijageneze.

2.5. ZAKLJUČCI

Obradi masovnih grobnica treba pristupiti temeljito, uzimajući u obzir svaki detalj prilikom prikupljanja podataka, ekshumacije i kasnijih postupaka u sali i laboratoriju. Predloženi algoritam objedinjuje navedene postupke, a posebno je važno istaknuti da svim postupcima treba rukovoditi ili ih nadzirati adekvatno educirani stručnjak. Analiza DNK predstavlja ključnu i danas najtočniju metodu identifikacije, dok su klasične metode značajno ograničene količinom i kvalitetom prikupljenih antemortalnih podataka, a u slučajevima obrade grobnica Iz Drugog svjetskog rata rijetko dovode do identifikacije. Antropološkim mjerenjima fragmenata bedrenih kostiju nije moguće pouzdano formirati parove koji pripadaju istoj osobi. Također, u istu svrhu ne mogu poslužiti niti rezultati denzitometrije. Rezultati denzitometrije ne pokazuju pozitivnu korelaciju s kemijski određenim koncentracijama kalcija i fosfora u ekshumiranim kostima, što je posebno izraženo za kalcij. Tumačenja rezultata denzitometrije i koncentracija mineralnih elementa iz ekshumiranih kostiju moraju biti u kontekstu s analizom tla i drugih okolišnih faktora, zbog utjecaja dijageneze.

2.6. SAŽETAK

Cilj ovog istraživanja bio je dati prijedlog Algoritma za postupanje i obradu koštanih uzoraka ekshumiranih iz masovnih grobnica. Prikazan je postupak ekshumacije, forenzičko-antropološki nalaz skeletnih ostataka žrtava iz masovne grobnice iz Drugog svjetskog rata, smještene na otočiću Daksa pokraj Dubrovnika. Urađena je DNK analiza radi identifikacije žrtava. Predložen je cjeloviti Algoritam postupaka prilikom obrade masovne grobnice.

U svrhu istraživanja mogućnosti reduciranja broja uzoraka za DNK analizu ispitana je upotrebljivost fizikalnih mjerenja fragmenata bedrenih kostiju radi simulacije sparivanja bedrenih kostiju iste osobe. Za razliku od sparivanja čitavih bedrenih kostiju iste osobe na temelju duljine i promjera glave femura, simulacija sparivanja bedrenih kostiju na temelju fizikalnih mjerenja fragmenata uzetih s istih područja kosti, nije dalo zadovoljavajući rezultat. U istu svrhu ne mogu poslužiti niti rezultati denzitometrije.

Uspoređeni su rezultati dobiveni primjenom denzitometrije s rezultatima kemijskog određivanja sadržaja kalcija i fosfora u koštanim ostacima. Rezultati denzitometrije ne pokazuju pozitivnu korelaciju s kemijski određenim koncentracijama kalcija i fosfora u ekshumiranim kostima, što je posebno izraženo za kalcij. Tumačenja rezultata denzitometrije i koncentracija mineralnih elementa iz ekshumiranih kostiju moraju biti u kontekstu s analizom tla i drugih okolišnih faktora, zbog utjecaja dijageneze.

2.7. SUMMARY

MULTIDISCIPLINARY APPROACH IN TREATING SKELETAL REMAINS EXHUMATED FROM A MASS GRAVE

The aim of the study is to submit a proposal of an Algorithm, created for treating and processing of bone samples from mass graves . We have presented the process of exhumation, phorensic–antropology findings of skeletal remains from the WWII mass grave. The DNA analysis was performed to identify the victims. Presented is a comprehensive Algorithm for procedures in mass graves treating.

To explore the possibility of reducing the number of samples for the DNA analysis, we examined the usability of physical measuring of femoral fragments to simulate femoral pairing of the same person. Pairing whole femurs of the same person based on length and the head diameter was successful, whereas the simulation of pairing based on physical measurements of bone fragments did not provide satisfactory results. The DXA scanning results cannot be used for this purpose, either.

The DXA scanning results were compared to the results of chemically determined content of calcium and phosphorus and do not show a positive correlation, particularly for calcium. Due to the effects of diagenesis, the interpretation of the DXA scanning results and concentration of mineral elements of exhumed bones must be taken in the context of the soil analysis.

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3. PRESLIKA RADOVA

3.1. Discovering the 60 years old secret: Identification of the World War II mass grave victims from the island of Daksa near Dubrovnik, Croatia

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Discovering the 60 years old secret: identification of the World War II mass grave victims from the island of Daksa near Dubrovnik, Croatia

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Aim To describe the organization, field work, forensic anthropological examination, and DNA analysis conducted to identify the victims from a World War II mass grave found on the Dalmatian island of Daksa near Dubrovnik (Croatia) in 2009.

Methods Excavation of the site was performed according to standard archeological procedures. Basic anthropological examination was made to determine the minimum number of victims, sex, age at death, and height. The bones with pathological and traumatic changes were identified. DNA was extracted from powdered bones and relatives' blood samples. Y-chromosome and autosomal short tandem repeats (STR) were used to establish the relationship of the remains with the putative family members.

Results The remains were found to belong to at least 53 distinctive victims. All were male, mostly with gunshot wounds to the head. DNA analysis and cross-matching of the samples with relatives resulted in 14 positive identifications using the Y-chromosomal STRs and 4 positive identifications using the autosomal STRs.

Conclusions This study showed that even in cases of more than 50-year-old, highly degraded human remains from mass graves, Y-chromosomal and autosomal STRs analysis can contribute to identification of the victims.

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After the partisan (communist-led) forces had entered Dubrovnik on October 18, 1944, mass executions followed, among them the execution on the nearby island of Daksa (Figure 1 and 2). According to historical sources (1), the execution took place on October 25, 1944 and the following days. According to the verdict issued by the "Court of the Military Command for the South Dalmatian Region" and announced on a poster on October 29, 1944, 36 Dubrovnik citizens, most of them prominent intellectuals, were "sentenced to be shot by a firing squad." The same historical sources do not state whether a trial was held or if there was a court under such a name (1).

Figure 1.



Map of Croatia showing its position in Europe and Dubrovnik with its nearby island of Daksa.

Figure 2.



Map of the island of Daksa showing two sites of the mass grave, Location 1 and Location 2.

In forensics, if the recovery of skeletons takes places a long time after death the possibilities to obtain information about victims' clothing, personal belongings, sex, age, height, and other characteristics may often be reduced. In such cases, DNA typing techniques may provide useful information. Y-short tandem repeat (STR) markers are paternally inherited and allow identification of male missing persons when the only available reference is a male paternal relative. The autosomal STR DNA profiling was established by using the commercially available miniSTR assay, which proved useful to obtain information from ancient and degraded DNA samples and was used in cases when the only available reference was a missing person's daughter (2). The DNA technology, including both STR analysis and mitochondrial DNA analysis, was already confirmed as a method of choice in the identification of missing persons in the 1991-1995 war in Croatia (3,4).

The aim of this study was describe the organization, field work, forensic anthropological examination, and DNA analysis by chromosome and autosomal STRs conducted to identify the victims from a World War II mass grave on the island of Daksa.

MATERIALS AND METHODS

The field work took place over fewer than 5 working days. During that time (including the time of departure/return from the island), the site was marked and trial trenches were dug and two locations treated (surface larger than 80 m^2, more than 150 m^3 of the excavated material) (Figure 3). Approximately 10000 bones and skeletal fragments, and a number of items were discovered, packed, and transported (Figure 4). In such circumstances, in most cases it was impossible to determine with certainty the body position and their interrelations.

Field work

The exhumation of mortal remains on the island of Daksa started on September 24, 2009, pursuant to the Order of the County Court in Dubrovnik. According to the historical data (1), it was assumed that the location of the mass grave was in the ruins of the former farm building basement. Another mass grave was also believed to be in the immediate vicinity. The second location was discovered about 15 m away from the first location.

Location 1 was an area surrounded by remains of a wall. It was divided, labeled, and staked out by sticks and ropes

into 55 equal fields measuring approximately 1×1 m. The surface layer of the soil was removed by a bulldozer. Bones started to emerge at the depth of approximately 1 m from the surface (Figure 5). Due to a large number of mixed and separated bones in different positions, intermingled with large stones, it was decided to give up the skeleton presentation in situ. This primarily referred to the Location 1, fields labeled "d." The bones were carefully excavated. After having defined the orientation (cranial/caudal) and the position related to the marked fields and the layer depth, the bones were packed in labeled cardboard boxes.

Location 2 was not staked out as Location 1, but was shaped as the excavation went on. Three parallel trenches were dug and marked as 2, 3, and 4, and divided into 9 equal fields measuring approximately 1×1 m. The trenches followed the direction of cracks in limestone. The findings of the excavation were the same as those at Location 1. The work was additionally obstructed by thick pine roots. Various items were found along with the bones at both locations: fragments of priestly collars, rosaries, buttons, necklaces, bullets, and shells (Figure 6). The excavation went on up to the hard layer at the depth of about 2 m.

Figure 3.



Mass grave site.

Figure 4.



Mass grave exhumation.

Work at the pathology room

The remains were transferred to the Department of Pathology of the Dubrovnik General Hospital, where they were cleaned and sorted out. Where possible, the bones were placed in anatomical position and analyzed to determine the age, sex, height, and changes that had occurred during lifetime or after death, and photographed. Samples for the DNA analysis were taken subsequently. After having taken measures of thighbones and head diameters, the height of a person was determined by the Trotter equation for calculating the body height (5).

Figure 5.



Human skeletons in the mass grave after the surface layer of the soil was removed by a bulldozer.

Figure 6.



The objects found at the site.

TABLE 1. Archeological data from the mass grave at Daksa Island, Location 1. The data are divided according to marked fields, see Figure 7

Field	Scapula	Clavicula	Humerus	Ulna	Radius	Ribs	Sternum	Vertebrae	Femur	Pelvis 1/2	Sacrum	Tibia	Fibula	Small bones	Skull
Da	2	2	2	2	2	20	1	20	1	1	0	3	4	82	1
Ea	3	3	3	4	3	29	2	36	5	5	3	3	3	155	1
Fa	3	3	3	2	3	33	1	32	0	1	1	0	0	80	2
Ga	0	0	0	0	0	1	0	4	2	0	0	4	4	97	2
Ha	5	6	6	6	6	84	3	59	6	6	3	2	2	176	0
Ia	0	0	0	0	0	0	0	6	2	0	0	3	4	68	1
Ja	2	2	2	1	1	19	1	28	1	4	2	0	0	63	1
Ka	2	2	2	2	2	17	1	19	0	0	0	0	0	26	1
Ib	0	0	0	0	0	0	0	0	0	0	0	2	1	15	0
Jb	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0
Total	17	18	18	18	18	203	9	204	18	17	9	18	18	762	9
Fc	0	0	0	0	0	7	0	7	4	3	1	5	6	0	0
Gc	4	4	3	1	1	31	1	23	1	3	2	1	1	81	1
Hc	7	4	6	6	6	52	5	63	10	6	6	3	3	200	1
Ic	3	3	5	3	4	33	2	33	1	4	2	0	0	93	2
Kc	5	4	5	6	5	75	2	58	0	3	3	0	0	188	2
Fd	0	0	0	0	0	0	0	1	4	1	1	3	3	13	0
Gd	4	4	6	7	6	81	4	58	8	7	1	14	14	281	3
Hd	4	5	3	3	5	59	2	69	3	4	2	4	4	175	3
Id	7	7	7	9	8	60	2	71	3	3	1	12	13	428	5
Jd	4	4	3	2	3	68	2	51	12	6	4	4	2	86	2
Kd	6	7	8	6	8	62	3	65	0	3	0	0	0	173	4
Total	44	42	46	43	46	528	23	499	46	43	23	46	46	1718	23
Overall	61	60	64	61	64	731	32	703	64	60	32	64	64	2480	32

TABLE 2. Archeological data from the mass grave at Daksa Island, Location 2. The data are divided according to marked fields (No. 2-5), see Figure 8

Field	Scapula	Clavicula	Humerus	Ulna	Radius	Ribs	Sternum	Vertebrae	Femur	Pelvis 1/2	Sacrum	Tibia	Fibula	Small bones	Skull
2	2	3	4	1	3	23	1	31	3	3	2	2	2	25	2
2a	2	3	3	4	4	35	2	43	5	5	2	5	4	75	0
2b	2	2	1	2	2	27	1	23	0	0	0	4	6	231	2
2c	2	0	2	1	1	4	0	13	4	10	5	7	6	28	0
2d	3	3	3	3	3	57	2	55	6	1	0	0	0	35	2
2e	6	5	3	3	3	37	3	35	0	0	1	0	0	76	2
2f	1	3	3	4	4	27	1	34	2	1	0	2	2	89	2
Total	18	19	19	18	20	210	10	234	20	20	10	20	20	559	10
3	2	3	2	2	2	14	0	21	2	2	1	6	8	181	3
3a	4	3	3	4	3	37	2	40	7	2	1	6	4	69	0
3b	2	1	3	3	4	36	2	28	5	10	4	1	1	115	1
3c	6	7	6	3	3	68	3	66	0	0	1	1	0	57	2
3d	0	0	0	2	2	4	0	9	0	0	0	0	1	17	1
Total	14	14	14	14	14	159	7	164	14	14	7	14	14	439	7
4	6	6	5	3	4	69	3	36	4	5	2	2	1	67	2
4a	0	0	0	1	2	0	0	6	2	0	0	4	5	84	1
Total	6	6	5	4	6	69	3	42	6	5	2	6	6	151	3
5	0	0	1	1	0	0	0	0	0	0	0	1	0	3	1
Overall	38	39	39	37	40	438	20	440	40	39	19	41	40	1152	21

Boundary values for the sex determination, based on the long bone length, were determined as described by Šlaus et al (6). Where possible, sex was determined by pelvic bones and skull characteristics. Due to the significant level of damage, this was generally not possible. Also, because of a large number of bones in a limited area and in the same layer, it was impossible to determine with certainty the body parts that belonged to the same person.

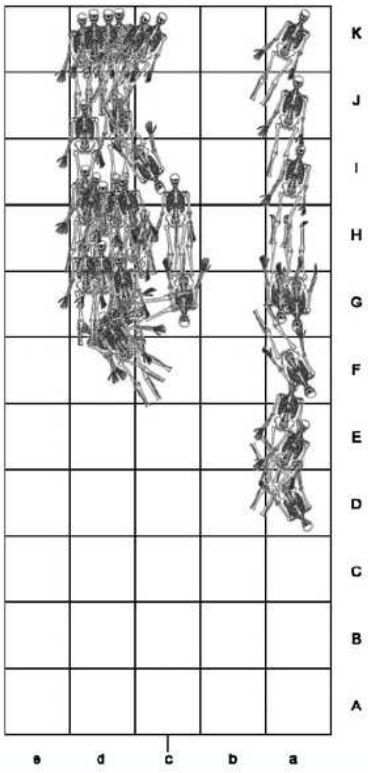
The age was determined by the pelvic articular surface (symphysis) following the Suchey Brooks method (7). Since most of the bones were badly damaged, a reliable determination was generally not possible. The location and the area of discovery and the total number of the bones discovered are shown in Table 1 and Table 2. Skeletal remains were identified and classified according to macro-sites (Location 1 and 2) and micro-sites (marked fields-quadrants) in which they were found (eg, Location 1 field Da). Fields in which skeletal remains were not found are not listed in the tables.

We made a schematic presentation of the assumed position of a particular body in relation to other bodies and their spatial orientation within the labeled schematic site (Figure 7 and 8).

DNA analysis

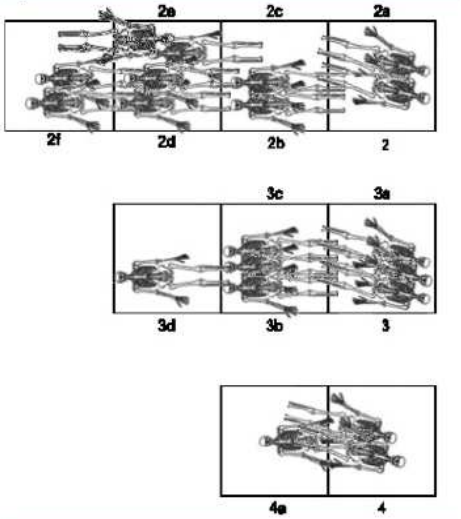
Prior to the DNA extraction, the bone/tooth surface was cleaned by abrasion with a grinding tip and sandpaper, the bone/tooth was crushed into small fragments, and stored in sterile polypropylene tubes at -20°C until analysis. After this initial treatment, the DNA extraction was performed as described by Alonso et al (8). At least two independent extractions were done (one or two craniums that presented teeth were sampled, as well as different postcranial bones like femurs). The DNA from blood and bloodstain reference samples of the living relatives was extracted by standard Chelex 100 protocols (9). The polymerase chain reaction (PCR) amplifications were performed on Parkin-Elmer thermal Cycler 9700, using the AmpFISTR Yfiler PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA) and MiniFiler PCR Amplification Kit (Applied Biosystems) (10,11). Typing of PCR products was performed on the ABI

Figure 7.



Schematic presentation of the finds at Location 1.

Figure 8.



Schematic presentation of the finds at Location 2.

Prism 310 Genetic Analyzer (Applied Biosystems) with the Data Collection Software. Electropherogram data were analyzed with the GeneMapper ID Software, version 3.2 (Applied Biosystems). The internal standard was Liz-500 (Applied Biosystems).

Analysis of typing results

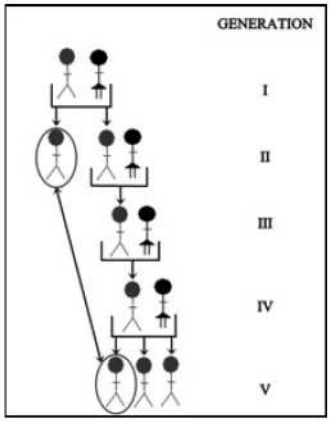
DNA profiles from skeletal remains were analyzed and compared with DNA profiles of the living relatives. The database was kept in the Microsoft Access 2000 (Microsoft, Seattle, WA, USA). Microsoft Excel 2000 (Microsoft) was used for statistical calculation. Calculation for statistical probability of biological relationship was performed according to standard protocols and the data from the Y Chromosome Haplotype Reference Database (YHRD) database (2,12,13).

Figure 9.



Gunshot wounds in the occipital region of a victim.

Figure 10.



Schematic presentation of positive identification in a case where great-grandfather's brother was identified by matching with great-grandson's paternally inherited Y chromosome.

RESULTS

Human remains were found at two sites designated as Location 1 and Location 2, and the skeletal remains belonged to at least 53 persons (Table 1 and 2). It can be stated with certainty that the bones were human and more than 50 years old, the time corresponding to the World War II. The skeletal remains had distinctive male anthropomorphic characteristics. Due to the bone damage of the remains, the age of a certain number of persons could not be determined.

Many of them had metal fillings, gold, and silver teeth and dental bridges and dentures.

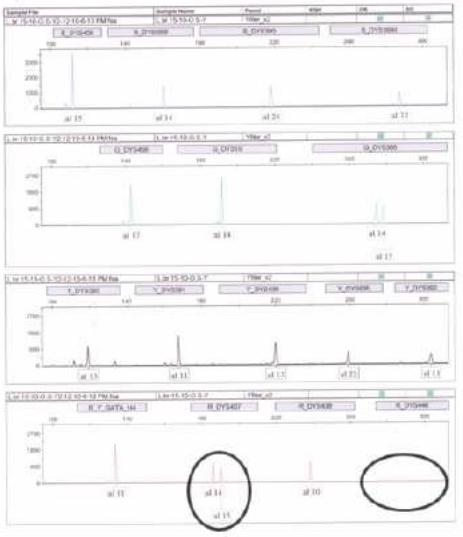
Gunshot wounds were identified in 22 persons. Practically all identified entry gunshot wounds were localized in the occipital region (Figure 9). One gunshot wound in the occipital region was identified in 15 persons, 2 gunshot wounds in 5 persons, and 3 gunshot wounds in 2 persons. Only one gunshot wound was identified in an area other than the skull, ie, the hip socket. Many skulls were found in fragments with peri-mortem fractures, although the head gunshot wound was not found. A tibia bone with a peri mortem fracture was also found on the site.

DNA was successfully extracted from 49 out of 53 skeletal remains (Table 3). In each case of tooth and bone samples, several DNA extractions were performed. AmpFlSTR Yfiler PCR Amplification Kit produced 34 out of 49 profiles, 32 of which were full profiles and 2 partial profiles. MiniFiler PCR Amplification Kit produced 40 out of 49 DNA profiles and only 2 were partial. Identifiler PCR Amplification Kit was used for 4 skeletons, and 2 full and 2 partial profiles were

TABLE 3. Summary of DNA analysis data for the skeletal remains from the island of Daksa

Characteristics	
Exhumed remains	53
Obtained DNA profiles	49
Ratio of obtained profiles (%)	92.43
Relatives' samples	23
Positive identifications	18
Ratio of positive identifications (%)	33.96
Ratio of positive identifications (%) regarding the number of requesting families	78.26

Figure 11.



Y chromosome short tandem repeat loci mutations in a father/son pair with double peaks at DYS437 locus and with null allele at DYS448 locus (in circles).

obtained. DNA was also extracted from 23 blood samples of the living relatives.

Cross-matching of the results with the relatives' data resulted in 18 positive identifications. In 4 cases, the victims were identified by using only the autosomal STR systems for identification because positively identified remains belonged to the fathers of the daughters who gave the blood sample. The results obtained by MiniFiler PCR Amplification Kit were confirmed by the Identifiler PCR Amplification Kit, which allows simultaneous amplification of 15 autosomal STR loci, whereas MiniFiler PCR Amplification Kit gives the profiling result for 8 autosomal loci (both kits include sex-determining locus amelogenin).

In other 14 cases, the victims were identified by the Y-chromosomal STR system because the victims were paternally related to the individuals who gave blood samples. Nine positive identifications were made for father/son pairs, 3 for nephew/uncle pairs, 1 for grandchild/grandfather pair, and 1 for great-grandson/great-grandfather's brother pair (Figure 10). We also found rare Y chromosome STR loci mutations in a father/son pair with double peaks at DYS437 locus, as well as with the null allele at DYS448 locus observed in the same father/son pair (Figure 11).

DISCUSSION

This study presents the anthropological, forensic, and genetic analysis of victims from the mass grave on the island of Daksa, near Dubrovnik. The remains were found to belong to at least 53 distinctive victims. All were male, mostly with gunshot wounds to the head. DNA analysis and cross-matching of the samples with relatives resulted in 14 positive identifications using the Y-chromosomal STRs and 4 positive identifications using the autosomal STRs. Forensic analysis revealed that most persons with identified gunshot wounds to the head died a violent death due to those wounds. The same applies to the persons with identified peri-mortem skull fractures and fragmented skulls. Arm bones and fore-arm bones tied up with wire indicate that this was a mass execution grave. The manner of the burial and particularly the localization of the penetrating gunshot wounds of 9 mm in diameter and the 9 mm pistol bullets with corresponding shells found at the site also suggest that this was an execution. The persons executed were probably in the kneeling position at the burial site or in its immediate vicinity. The objects found at the site indicate that they were mostly civilians. Also, according to the items found in the mass grave and to the results of DNA identification, at least 3 victims were priests.

One of the main problems in excavations of the mass graves dating from the World War II is a large number of damaged and separated skeletal remains (14). The excavations are usually complicated by environmental factors, such as difficult access to the site, subsequently raised constructions, vegetation, and often only assumed micro-location of the gravesite (15). Financing, manpower issues, the available time, and particularly the lack of experienced and educated teams also have a significant impact on the methodology of excavation.

The main objective of the World War II mass graves exhumation is to determine the minimum number of victims (if not always the precise), identify the cause and manner of death, and carry out the identification test, if possible, and return the remains to their families, in accordance with the Geneva conventions (16).

Since the bones of a large number of victims were found separated and intermingled, often in piles, a fast and bulk approach is satisfactory, particularly if the grid

use (network) and the orientation of long bones at the site is carefully observed. Such method provides a significant saving of time and resources and still gives necessary answers to the questions raised. With the methods described above and additional work in the pathology room and at the desk, it is possible to subsequently reconstruct positions and relations of the remains by combining the data collected during the exhumation with the basic anthropometrics data revealed in the pathology room.

Anthropometrics data and the teeth status have an extremely limited role in the identification of mass graves victims dating from the World War II, mostly because there are no ante-mortem data related to victims (17,18). There was a relatively limited number of citizens of Dubrovnik who were expected to be found on the island of Daksa, but after more than 60 years no relevant data on their physical characteristics (more accurate than short/tall) or the teeth status could have been obtained, although a large number of victims had significant prosthetics works. There were no significant problems in the anthropological determination of the victims' sex, whereas the age determination (especially for the older ones) was not sufficiently precise for identification. We were able to determine the actual age of the victims only after the DNA analysis and positive identification, according to the information given by the relatives (19).

It was easy to identify the cause and the manner of death, since the execution with a single shot to the back of the head was a common manner of execution in mass graves victims from the World War II discovered in this region, as was the burial at the place of the execution (17).

A well-organized field work and careful proceedings in the pathologist room can "tell the story" about the last moments of the victims' lives, while the identification of victims is exclusively in the domain of the DNA analysis (14,17,20). The latter was the imperative in our research, and was mostly done through Y chromosome analysis of male skeletal remains.

We successfully identified 14 persons by the AmpFISTR Yfiler PCR Amplification Kit. One of them was an honorable Croatian priest, reverend Petar Perica, known as the author of some of the most beautiful Croatian religious songs (Box 1). His identification was confirmed by the Y-chromosome STR profile compatibility with the blood sample of his brother's great-grandson.

While observing a null Y-STR locus should not represent a problem for the profile interpretation, more than one peak at one or more loci could lead to a grave misinterpretation of a profile and its sample source. The same rare mutations and their interpretation have been already reported (21). Multiple variations (deletion or duplications), even if rare, can happen in the same single-source sample and these examples should be a reminder before drawing premature conclusions. Forensic interpretation of Y-haplotype profiles should be in the focus because multiple alleles at various loci do not necessarily indicate that the sample originates from a mixture. This also showed the importance of the DNA identification methods and their application in the case work.

This study showed that even in cases of more than 50-year-old, highly degraded human remains from mass graves, Y-chromosomal and autosomal STRs analysis can contribute to the identification of the victims.

BOX 1. Croatian religious poem written by Rev. Petar Perica, one of the identified victims

Heavenly Virgin, Queen of the Croats

(Rajska djevo kraljice Hrvata)

Hail, Mary, full of all graces,
Eternal sunshine, clad in radiance,
Circling your brow, a starry crown,
Below your feet, hell's dragon groans.
Heavenly Virgin, Queen of the Croats,
Our Mother, Our Golden Dawn,
From devoted hearts, receive a gift,
Receive our pure and fervent love.
Blessed are you, all Immaculate,
The serpent's breath taints not your breast!
Star of happiness, resplendent for us,
Disperse evil darkness, nights of sin!

http://www.youtube.com/watch?v=I_SC8_SGHv0

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Declaration of authorship IB participated in the research and in writing of the manuscript. DS participated in the field work and organized the writing of the manuscript. JLJ contributed to the experimental part of the DNA analysis and to the writing of the manuscript.

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3.2. Identification process of skeletal remains from mass graves: our experience and proposal guidelines

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Identification process of skeletal remains from mass graves: Our experience and proposal guidelines

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ABSTRACT

Aim of this paper is to present our experience and proposal guidelines for reducing the number of samples for DNA analysis of skeletal remains from mass graves, whether for scientific purpose or for the identification of mass graves victims. Therefore, the analysis of 94 bone fragments included the following measurements: femur length and the femoral head diameter, the diameter of the upper, wider portion and lower wider portion of the bone fragment, densitometry of the fragments and measurement of mass and volume of fragments. Bone density was determined on the basis of measured values of mass and volume. The results of fragment matching by physical analyses were compared with the pairing results obtained by previously conducted DNA analysis. Deviation in measured values of matching bone fragments that made a pair was calculated for all successfully matched fragments. By the results of DNA analysis 36 femoral pairs were successfully formed. Measured values were added to the DNA analysis. Out of 36 pairs, positively ascertained by the DNA analysis, 29 pairs were formed after adding the results of physical measurements and removing the data where femur samples were damaged. Total correspondence in measurements of the femoral length was noted in 25.9% pairs, while the correspondence within the 5% error was 100%. Density of the tested femurs was significantly different for the same person (DNA match), both for the left and the right femoral fragment. It would be optimal to choose only the whole-length left or right femur and thus reduce the number of samples by 50%. With regard to the results of our research and the observations deriving from them, as well as to the guidelines we used in the study, we suggested these guidelines be used both for scientific researches and to identify mass graves victims.
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1. Introduction

Man has always asked questions about the essence of his existence and the secret of life he has been holding. The need to understand urges him to do continuous researches, incites philosophical and religious debates and scientific objectification of the macro and micro world. Identifications of human remains are often time consuming and complex processes. Methods of identification should be planned, diverse and complementary to increase the number of efficient identifications and fulfill the main objective - to show respect for the deceased and return them to their families [1,2]. The protection of human rights of both living and deceased persons, will be supported and passed on to next generations in this way.

One of the major achievements of molecular biology in forensic medicine is the identification by DNA typing of biological samples. This particularly relates to the identification based on bone or teeth samples taken from the people killed in mass disasters or from the exhumed bodies, which cannot be performed by any other standard method [3–8]. The DNA analysis is a very useful and certainly the most precise current method of identification in such cases. The analysis is based on the comparison of the DNA. Human DNA, isolated from skeletal samples (bones or teeth), is compared to the DNA isolated from blood samples, buccal mucosa swabs, hair and the like, taken from the presumed closest relatives [3,9].

There is a number of published researches dealing with the problems of identification of victims found in mass graves after the Homeland War, World War II and even after World War I [3–8,10]. Due to the difficulties of isolating DNA from such samples,

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the majority of authors treated the problem of the DNA isolation while only a small number engaged in the proper selection of number and type of bone samples for the DNA analysis.

The success of any forensic identification depends to a large extent on the range and preservation of the data collected in the field [3,11]. Warring parties use different methods to hide war crimes and the location of the burial: excavation and relocation of remains from one place to another; further relocation to tertiary sites; disassembling and mixing of parts of the body, compacting and crushing. All such activities complicate or make it impossible to determine the number of bodies, their assembling and identification [3,8,12]. Consequently, the number of fragmented bone samples is dizzily rising. The cost price of the DNA analysis would be extremely high in such situations and the DNA identification could not be made for all samples.

The ideal situation with preserved skulls, teeth and the corresponding number of femur pairs is rarely found. If the identification is performed only on the basis of the femur it seems logical to halve the number of samples and restrict the DNA analysis on left or right femurs only. Although it is relatively easy to morphologically distinguish larger fragments of the left from the right femur, the problem arises when some of the femurs are missing (either the left or the right one) or if they are fragmented to such an extent that they are not comparable.

Our idea was to explore the possibility of distinguishing and matching femurs of the same person on the basis of their size and physical properties after they were previously DNA analyzed and paired (left/right) with certainty. Therefore, we checked the usability and features offered by available and not overly expensive physical analyses used in the process of pairing left and right femur fragments from which samples would be taken for the confirmatory femur pairing DNA analysis.

The second objective of this study was to present guidelines/standardization of skeletal remains from mass graves based on our experience.

2. Experimental

All studies were approved by the Ethical Committee of the University of Split School of Medicine (No. 32-1/06). In the study we analyzed the femurs found in bone fragments, exhumed in mass graves on the island of Daksa, near Dubrovnik, in September 2009. They were previously linked by the DNA analysis and some of them were identified [13]. Correspondence between bone fragments (for all successfully matched bone fragments) has been expressed by statistical calculation as the measure of success of physical analyses in pair-forming. The result is the optimization suggestion for the DNA analysis of the bone samples number.

The treatments of the mass grave and positive identifications have been presented in our previously published study [13]. The location was processed complying with standard archeological proceedings. Basic anthropological tests were performed to determine the minimum number of victims, their gender, height and age at the moment of death. Bones with pathological and traumatic changes were identified. The DNA was extracted from bone samples and from blood samples of the presumed relatives. To determine relationship between the victims and their potential relatives we used the AmpFlSTR Yfiler PCR Amplification Kit, and MiniFiler PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA).

2.1. Material

About 10,000 bone fragments were found and singled out at two locations: 53 skulls (32 in location I and 21 in location II); 104 femurs (64 in location I and 40 in location II). The treatment of 104 femur specimens was closely examined in the study. Considering the number of the skulls found, we assumed that there were 53 victims buried in the grave (all of them men). Femur samples were chosen for the analysis because of their solid structure. Because they are often well-preserved, the high quality DNA can be successfully isolated most of the time. The number of samples for the DNA analyses can potentially be halved by matching the left and the right femur and further analyzing only one femur per victim. The procedure of the whole treatment and physical analyses of femur samples is shown in Fig. 1.

2.2. Methods

After examining the bone fragments we created a database and measured the following values: femur length (L) and the femoral head diameter ($Ø$). In our previous study, we used Trotter equation for calculating the body height [13]. Very often we only have parts of the femur. In such situations, there is no reliable way to pair with certainty a left and a right femur by measuring. However, we tried to reduce the number of samples for further, more expensive analyses, by applying simple physical measurements. So we decided to make measurements on fragments excluded from equal positions, to simulate the situation in which we do not have the whole femur. Parts of the femur were then isolated at the 10% length of the total femur length and measured at the distance of 20% from the cranial end (Fig. 2). Measurements were performed on a personally constructed anthropometric table. Further analyses were made on these isolated fragments of the femur: measurement of the outer diameter of the uttermost bone fragments, physical values: weight, volume and surface density of bone fragment.

2.3. Outside diameter of the uttermost parts of femur fragments-measuring by caliper

Measuring was made by the caliper with an accuracy of ±1 mm and the measuring range of 0–40 mm. The following variables presented in millimeters (mm) were measured in all femur fragments: the diameter of the upper, wider portion of the bone fragment ($D1$); the diameter of the upper, narrower portion of the bone fragment ($D2$); the diameter of the lower, wider portion of the bone fragment ($D3$); the diameter of the lower, narrower portion of the bone fragment ($D4$) (Fig. 2).

2.4. Weight measuring of femoral fragments

The weight of all femur fragments is shown in grams (g) and measured by the analytical balance with accuracy ±0.1 mg. The values measured were then entered in a previously constructed database.

2.5. Density measuring of femoral fragments by densitometry

The bone mineral density (BMD) of all femoral fragments was measured by a quantitative method – densitometry. The BMD was expressed in grams per centimeter squared. The densitometer model QDR 4500 C (S/N 48 034; Bedford, MA 01730, USA), was used for this purpose and the measured values entered into the database.

2.6. Volume measuring of femoral fragments

The volume of all femoral fragments was measured by the laboratory cylinder with the measuring range up to 250 ml and expressed in cubic centimeters (cm^3). Fragments have been immersed in cylinder filled with distilled water and left for 24 h.

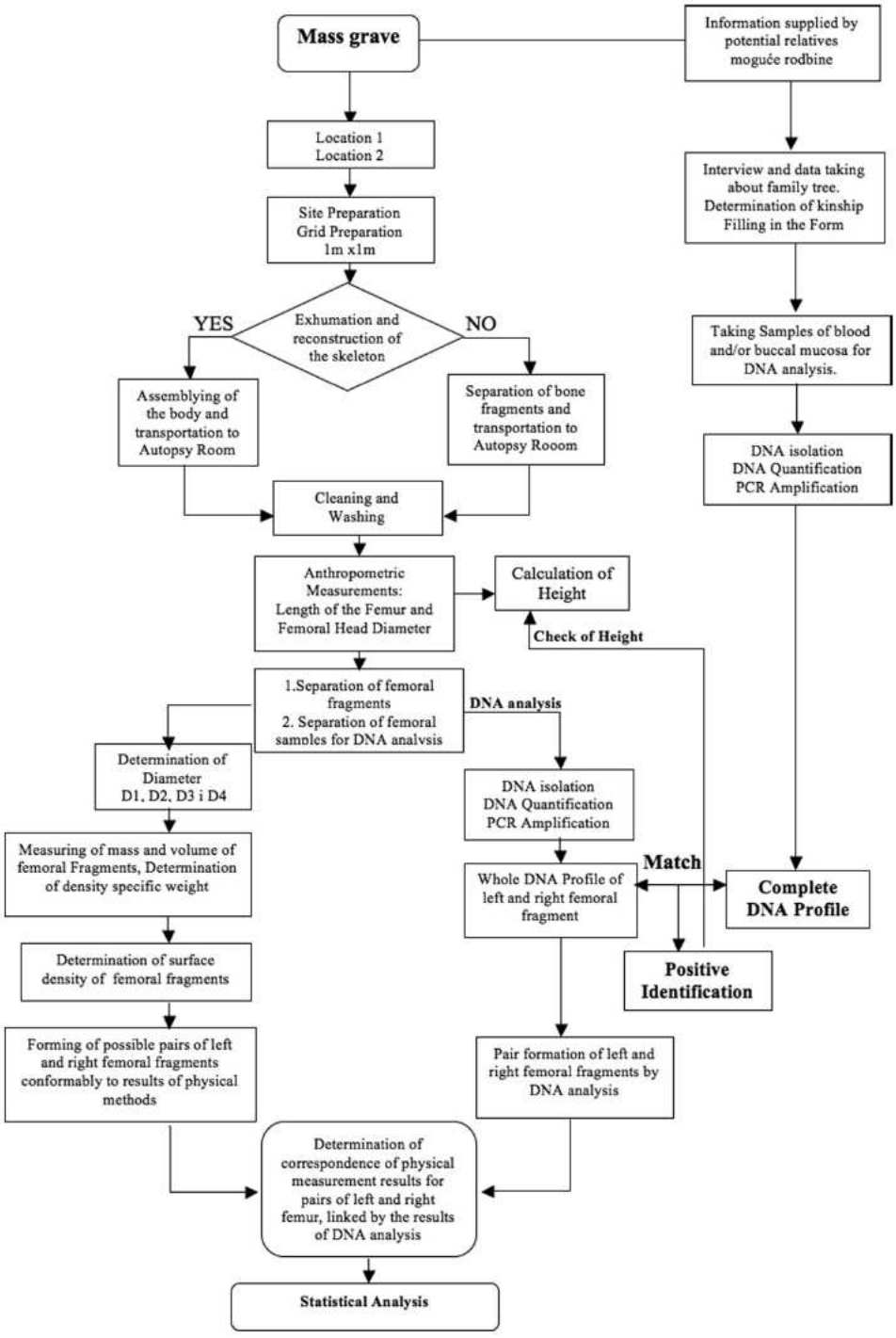


Fig. 1. The procedure of the whole treatment and physical analyses of femur samples.

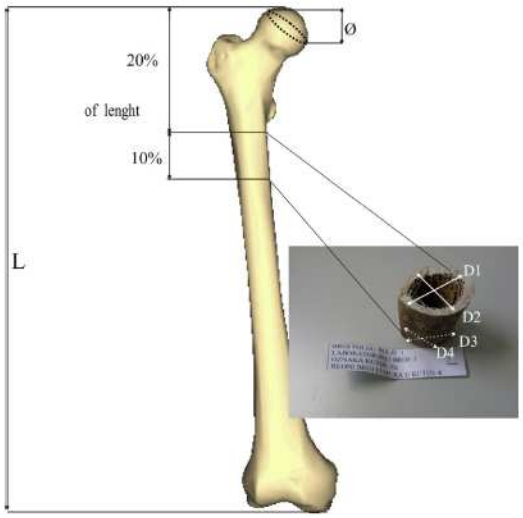


Fig. 2. Measurement positions for femur length (*L*), femoral head diameter (*Ø* and the diameters of bone fragments; upper wider portion (*D*1), upper narrow portion (*D*2), lower wider portion (*D*3) and lower narrow portion (*D*4).

The volume was measured three times: before immersion, after immersion and 24 h after immersion. Largest difference in the volume is taken as a bone volume.

2.7. Determination of average density of femoral fragments based on measured values of weight and volume

On the basis of measured values of weight and volume, average density values given in g/cm^3 were calculated for all femoral fragments by applying the mathematical expression for determining density: $\rho = m/V$ (ρ: density, m: weight, V: volume) and the Microsoft Excel 2010 software program. The measured values were entered into the database.

2.8. Pairing of femoral fragments

After measuring and calculating all the parameters, femur fragments were paired on the basis of the values recorded in the database: the exact location from where the fragments originated the outside diameter of the uttermost parts of bone fragments, weight, volume and density of the fragments, surface density of bone fragments. The results of the pair formation obtained by physical analyses were entered into the database and compared to the results of the pair formation based on the previously made DNA analysis.

2.9. Statistics

For all successfully paired bone fragments determined by the DNA analysis, concurrent deviation was determined by statistical calculation in values for measured parameters between bone fragments which form a pair. Continuous data were expressed as average, median and standard deviation (S.D.) The significance of the differences between values was tested using SPSS software 11.03. for Windows and MS Office Excel 2010 packages. *T*-test was used to calculate correlation between the measurements. For all statistical tests a significance level of 95%, ($P \leqslant 0.05$), was used.

3. Results and discussion

Since it was not possible to determine the DNA profile for all 104 femurs found (poor quality of the material), 94 femoral fragments were treated by physical analyses, which included different measurements. On the basis of the DNA analysis 36 femoral pairs were successfully matched (72 femurs) while others could not be matched because the number of loci for secure pairing was insufficiently amplified during the DNA analysis.

Measured values were added to the DNA analysis. Out of 36 pairs, positively ascertained by the DNA analysis, 29 pairs were matched after adding the results of physical measurements and removing the data where femur samples were seriously damaged and key measures could not have been taken.

To verify the efficiency of physical analyses in forming pairs of bone remains, differences were calculated between the left and the right bone fragment of a pair for 29 successfully matched pairs of femoral fragments in all measured values for all parameters (Table 1). Differences in measured values have been given in percentage as "concurrent deviation" between two values, i.e. between the values measured for one pair in relation to its pair. To facilitate the interpretation of differences between the values, the results were classified into four categories with regard to concurrent deviation: complete matching; concurrent deviation <5%; concurrent deviation between 5% and 15%; concurrent deviation >15% (Table 1). Correlation results obtained after applying the statistic *T* pair test for samples of the left and right femoral side was shown in Table 1.

The results of the analyses performed showed that pair matching (or excluding) of the left and right femur could be done with high probability by using only the data referring to femoral length and the femoral head diameter. The results obtained by measuring of the diameter of the left and right femur at two heights and two widths are not a reliable information in the formation of pairs. Specific gravity showed the lowest correlation factor of all examined parameters. To our surprise, superficial density of the femurs tested was significantly different for the same person (DNA match), both for the left and the right femoral fragment.

The length of the left and right femur differs in less than 5% of the value (a few millimeters). Therefore, it is possible to form as many femoral pairs, which most likely belong to the same person if skeletal remains are properly extracted and classified on the ground during excavation.

The smallest differences in measured values for the fragments, which make up a pair, were recorded in measuring the femur length and the femoral head diameter. Total correspondence in measurements of the femoral length was noted in 25.9% pairs, while the correspondence within the 5% error was 100%. Total correspondence in measurements of the femoral head diameter was noted in 51.7% pairs, while the correspondence within the 5% error was 96.6%. Consequently, the most reliable were the measurements performed on the entire femur as opposed to the measurements performed on femoral fragments. It is therefore obvious that only the whole femurs can be matched with high probability while physical measurements are not helpful in femur fragments pairing (especially distal parts). For future study samples we should consider the possibility that determining several different parameters on the entire femur would result in smaller differences of measured values in a presumed bone pair and provide higher degree of safety in femur pairing. Several researches have been carried out in Croatia in which long bones or their parts were used to get specific population standards for determining gender, but in

Table 1
Differences and compatibility (if differences = 0%) between measured values of the left and right femur (29 pairs): femur length (L); femoral head diameter ($Ø$); the diameter of the upper, wider portion of the bone fragment ($D1$); the diameter of the upper, narrower portion of the bone fragment ($D2$); the diameter of the lower, wider portion of the bone fragment ($D3$); the diameter of the lower, narrower portion of the bone fragment ($D4$) (see Fig. 2); average density (ρ) values and body mineral density (BMD).

Pair	Differences between measured values of the left and right femur: separately for each pair (%)							
	L	$Ø$	$D1$	$D2$	$D3$	$D4$	ρ	BMD
1	0.25*	−6.25†	3.39*	1.46*	0.28*	4.86*	1.28*	−0.15*
2	−0.46*	−2.17*	4.93*	−3.92*	−4.12*	−0.79*	−3.51*	−1.94*
3	1.73*	1.96*	2.62*	15.02‡	−5.06†	12.67†	7.59†	3.97*
4	1.09*	0.00	0.59*	−7.17†	−5.23†	−5.37*	12.71†	0.95*
5	0.00	0.00	0.00	1.38*	2.22*	4.21*	−11.41†	14.42†
6	0.22*	0.00	2.81*	−3.64*	−3.16*	1.43*	29.96‡	2.73*
7	0.00	0.00	−0.62*	1.57*	1.59*	−4.38*	0.00	13.24†
8	0.64*	0.00	−0.32*	3.45*	−0.65*	2.27*	−5.00†	7.50†
9	−3.27*	0.00	−2.87*	1.83*	3.63*	7.53†	−13.42†	−5.06†
10	−1.16*	−2.17*	−2.53*	4.00*	−5.59†	3.56*	−6.13†	4.80*
11	−0.72*	2.13*	5.26†	1.40*	1.72*	1.90*	−2.52*	2.07*
12	0.25*	0.00	−1.10*	1.13*	0.76*	0.00	−76.09†	0.77*
13	−1.04*	0.00	−0.32*	5.56†	−7.30†	−2.33*	−0.56*	−4.41*
14	0.00	0.00	5.99†	2.55*	8.94†	2.59*	3.35*	−0.93*
15	0.00	−2.08*	−10.14†	−1.52*	−9.09†	3.80*	4.85*	−4.55*
16	−0.65*	0.00	7.14†	−4.68*	9.76†	0.38*	8.18†	9.30†
17	−0.22*	2.00*	5.03†	6.29†	0.36*	6.59†	−25.32‡	3.36*
18	0.21*	−2.00*	3.51*	−0.73*	−1.02*	0.36*	15.20‡	−1.56*
19	0.00	−4.26*	5.96†	8.80†	10.14†	0.00	1.01*	−0.58*
20	−0.42*	1.92*	−3.19*	0.66*	0.97*	1.05*	5.57†	5.97†
21	0.23*	−2.04*	11.55†	−8.07†	−2.62*	0.00	6.32†	1.62*
22	0.00	1.89*	2.70*	−3.19*	−0.31*	−1.12*	36.26‡	2.13*
23	0.00	0.00	3.72*	4.81*	9.41†	2.43*	−0.59*	−5.90†
24	−1.11*	0.00	−0.72*	−1.67*	−0.74*	−0.42*	−5.05†	4.04*
25	−1.27*	0.00	−6.87†	5.63†	−9.12†	3.04*	−19.38‡	1.64*
26	1.79*	−3.77*	1.50*	4.98*	2.56*	1.95*	20.22‡	−0.53*
27	0.22*	0.00	0.00	0.68*	0.93*	1.37*	16.30‡	−3.29*
28	−0.23*	0.00	0.00	0.40*	3.38*	−6.56†	6.74†	−4.56*
29	1.74*	−4.35*	0.00	−1.54*	2.08*	3.35*	13.50†	1.54*
Differences and compatibility	Differences between measured values of total of 29 femur pairs (%)							
	L	$Ø$	$D1$	$D2$	$D3$	$D4$	ρ	BMD
Compatibility 100%	25.9	51.7	13.8	0	6.9	0	3.4	0
*Differences ⩽ 5%	74.1	44.8	58.6	65.5	72.4	69.0	27.6	51.7
†Differences between 5% and 15%	0	3.4	27.6	34.5	20.7	27.6	37.9	37.9
‡Differences ⩾ 15%	0	0	0	0	0	3.4	31.0	10.3
§Results of *T test* obtained after applying the test for samples of the left and right femoral side, i.e. femoral fragments ($N = 29$ pairs)	0.984	0.946	0.868	0.747	0.758	0.800	0.675	0.829

§ *T*-test is significant at the 0.01 level and 95% CI.

available literature we found no data referring to femur pairing analyses [14].

Apparently, teeth have priority as the sample source, but in situations where teeth or skulls are not found or when there are discrepancies between the number of skulls and other bones of exhumed persons, the femur is a logical choice as the sample source for DNA analysis.

Due to the fact that the femur is the strongest and the largest bone in the human skeleton and is often well preserved it provides the best source of samples for the DNA analysis [14]. We believe that possibility of pair matching could be useful in both practical and research work. The maximum length of the femur and the largest diameter of the femoral head are standard variables used in the discriminate-function analyses of femur to determine the gender and height of a person within physical anthropology and bioarchaeology analyses [14,15]. It has been shown in the study that the number of samples (and related costs) for the DNA analysis can be reduced by femur pairing without decreasing reliability of identification and the number of persons identified.

It would be optimal to choose only the whole-length left or right femur and thus reduce the number of samples by 50%. Unfortunately, since bone fragments are often found in mass graves and only a few whole-length femurs (either left or right), any method of reducing the number of samples necessary for the DNA analysis is welcome.

One of the most important characteristics of DNA technology is its originality and validity. The DNA analysis method is compared to the fingerprint analysis because it represents a unique, genetic profile, which is specific for each one of us [9].

With regard to reliability and usefulness of the results of the DNA analysis, the procedures and methods which precede it, physical analyses in the context of this research, reliability and accuracy is also expected. Due to the important application of the DNA analysis in identification procedures, it is obligatory to meet the highest standards from the moment a sample enters into the laboratory to processing and presentation of results to family members of the victims. It also implies that all the procedures preceding the DNA analysis should meet the highest standards. *Guidelines for International Forensic Bio-archeology Monitors of Mass Grave Exhumations* was published by Skinner et al. in 2003 [16].

It was stated in the work that the situation in a mass grave is always a huge challenge for anthropologists and pathologists. They warn about the situations when insufficiently competent ad hoc agencies and/professionals are engaged in the work with mass graves. In such situation major mistakes are possible, which often cannot be repaired.

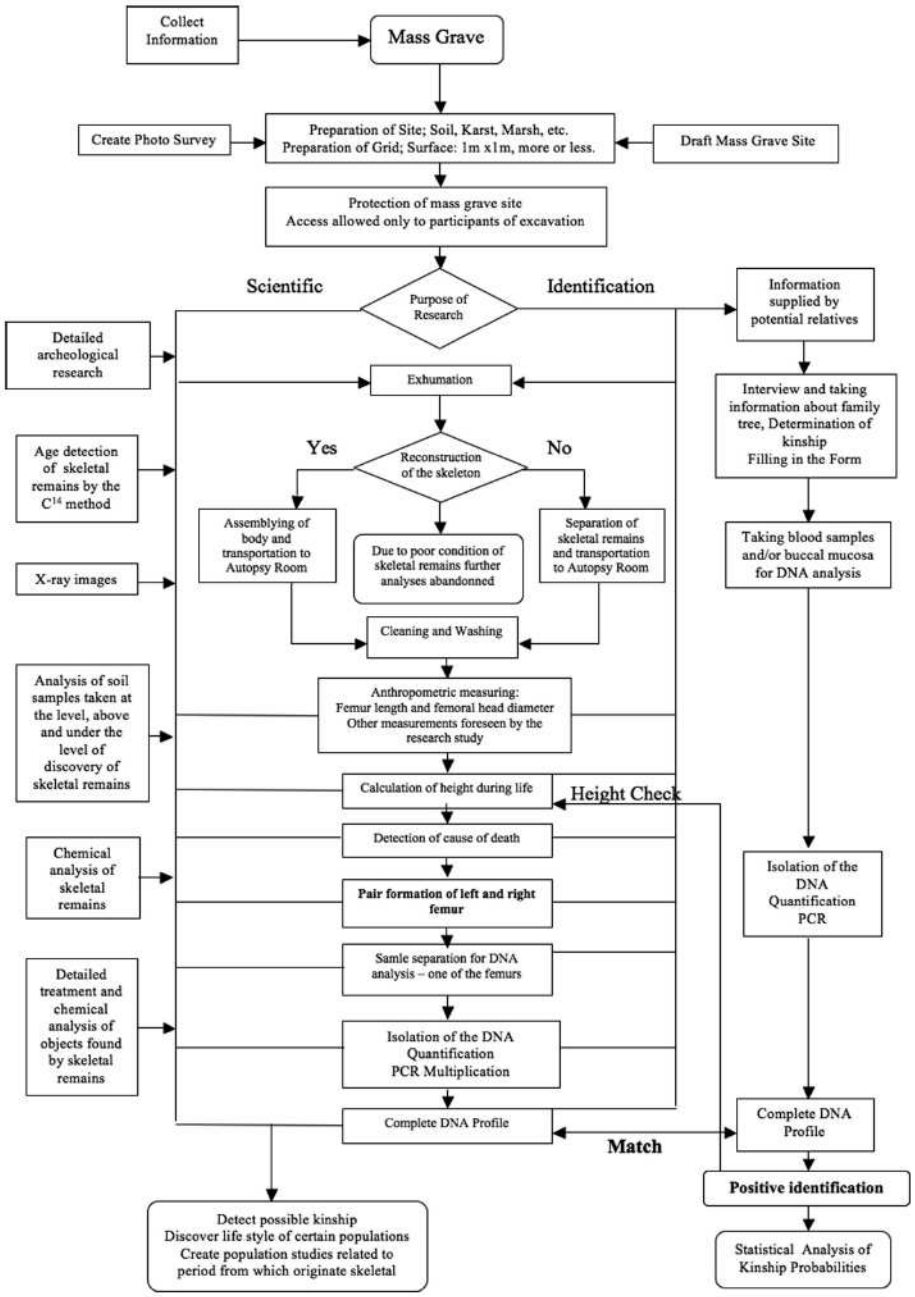


Fig. 3. Flow chart draft guidelines for processing of the mass grave samples.

It is necessary to develop purposeful methods (physical, chemical, biochemical and molecular) based on scientific knowledge's of anthropology to optimize the number of samples for the costly DNA analysis/testing. These complementary methods contribute to faster and better development of forensics and help to resolve the cases where efficiency would be questionable if only one of the two methods would be applied.

In 2007 Prinz et al. gave an overview in their study of treating the samples found in mass graves and suggested an interdisciplinary approach to obtain a greater number of identifications [17].

Definis and Sutlović described minutely in their study the experiences in identification of mass graves victims, with special reference to the identification of men/monks. Since they have no direct descendants and their parents are dead, the only way of identification was by applying the DNA analysis based on multiplication of the Y chromosome locus [4].

In conclusion, with regard to the results of our research and the observations deriving from them we suggested these guidelines to be used both for scientific researches and to identify mass graves victims (Fig. 3).

The suggested guidelines include a number of different procedures and/or analyses. If some of the analyses suggested are not performed in proper time they may not be completed afterwards (due to the field devastation, poor handling of exhumed samples, excluding samples for the DNA or other analysis, or some other actions). It is, therefore, extremely important to take care of the course of the entire procedure and perform each part of the analyses in due time. The choice depends on whether the treatment of skeletal remains is performed for scientific researches or for identification purposes. We believe that it is very unlikely that the carbon C^{14} analysis will be performed if the mass grave has been processed for identification purposes. A part of the guidelines aimed for scientific researches includes the carbon C^{14} analysis, X-ray recording, chemical analysis of skeletal remains and the surrounding soil, utensils and items found at the burial site. The suggested guidelines do not include taking of biological samples of the presumed relatives, which is included in the section relating to identification.

4. Conclusions

It should be noted that similar guidelines have not been found in available literature. In our opinion they can be useful to all those who are beginning to work with the DNA identification of mass graves victims, as well as to those who have years of experience in this work. Their experience can contribute to create even better guidelines necessary to standardize the processing of mass graves samples. Naturally, some paths are dead ends, but all possibilities which could bring positive results should be investigated and tested. Sometimes, instead of answers we get new questions.

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3.3. Bone mineral density of skeletal remains: Discordant results between chemical analysis and DXA method

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Bone mineral density of skeletal remains: Discordant results between chemical analysis and DXA method

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ABSTRACT

Dual-energy X-ray absorptiometry (DXA) scanning is a gold standard for bone mineral density measurement and diagnosis of primary and secondary osteoporosis in living persons. DXA is becoming widespread when analysing archaeological material, and is considered to provide an accurate diagnosis of osteoporosis in skeletal samples.

The aim of this study was to explain the differences in results between bone mineral density (obtained with DXA) and chemical determination of calcium and phosphorus concentrations in skeletal remains. We examined bone mineral density (BMD) and mineral content of femoral bone samples exhumed from mass graves of the Second World War. BMD was determined by Hologic QDR 4500 C (S/N 48034) Bone Densitometer. Concentrations of calcium and phosphorus were determined with AAS (Atomic absorption spectroscopy) and UV/VIS (Ultraviolet–visible) spectroscopy.

The results obtained in this study do not support the hypothesis according to which BMD measured by DXA scan has positive correlation with chemically determined concentrations of calcium and phosphorus in bones, especially in acidic soils where there was significant impact of diagenesis observed.

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1. Introduction

The DXA (dual-energy X-ray absorptiometry) technique uses X-rays of two different energies to determine bone mineral density (BMD, g/cm^2) and bone mineral content (BMC, g). The Dual-energy X-ray absorptiometry is considered as a gold standard in measuring bone mineral density and is most widely used diagnostic tool for primary osteoporosis (usually age related and associated with post menopause) as well as for secondary osteoporosis (osteoporosis caused by other conditions, such as hormonal imbalances, diseases or medications) [1]. In Croatia, the method is also used for that purpose, exclusively. However, previous research have shown that DXA is also becoming used from the aspect of forensic medicine in other parts of the world. Namely, several authors have used DXA scan for gender and age prediction [2,3], stress indication [4] and osteoporosis [5] of skeletal remains.

Densitometer measures the quantity of hydroxyapatite in bone, as bone mineral content (BMC) in grams, and calculates the areal BMD, expressed as grams of mineral per unit area scanned [6].

Mature bone is composed mainly of proteins and minerals. Approximately 60% of bone weight is mineral. Bone mineral content is in 80–90% composed of calcium and phosphorus, which form the inorganic component of bone matrix [7]. Calcium (Ca) and inorganic (i) phosphorus (P) are the two main constituents of hydroxyapatite, bone mineral that strengthens the mechanical resistance of the organic matrix. Over 99% of calcium and 85% of phosphorus in the human body exist as a complex within bone where the ratio between Ca/P mass is 2.2 [8].

Diagenesis is a natural process that alters the proportions of organic (collagen) and inorganic components (mainly hydroxyapatite) of bone exposed to environmental conditions. It is accomplished by the exchange of natural bone constituents, deposition

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in voids or defects, adsorption onto the bone surface and leaching from the bone [9]. Hydroxyapatite is insoluble in water, but it breaks down into soluble salts of calcium and phosphorus in the presence of an acid environment. If the soil is neutral or basic, a skeleton may persist for centuries in good condition. Mineral dissolution will also occur in a corrosive soil environment (irrespective of taphonomy) [10]. In order to understand post-mortem chemical changes in bone, geochemical conditions at a burial site must be taken into account. The most important variable for demineralisation and decomposition of hydroxyapatite in soil is pH, while several other factors such as organic matter content, mineralogy, and texture, soil solution fluoride and carbonate concentration, temperature regime, abundance and distribution of precipitation, local groundwater movement, microbial activity, and duration of interment can also affect the skeleton remains [11].

To our knowledge, no studies have yet been performed to assess BMD and correlation with calcium and phosphorus concentrations (and ratio) in human skeletal remains. Respectively, this is the first study to measure BMD with Dual-energy X-ray absorptiometry, and Ca and P content with Atomic absorption spectroscopy and Ultraviolet–visible spectroscopy (respectively) of bone samples exhumed from mass graves dating from World War II. Also, this is a novel research in Croatia, considering the use of DXA method from forensic aspect.

Our expectation was positive correlation of bone mineral density and mineral content (calcium and phosphorus) with the concentrations of calcium and phosphorus, but only the results obtained for phosphorus acted in predicted manner, while values of calcium were higher than expected.

2. Experimental

2.1. Sample collection and screening

Femoral samples found in mass graves are often not preserved as a whole, but partially fragmented. Fragments of the right and the left femur bones of middle aged men were excavated from mass graves at two locations during our previous studies [12,13]. The left and the right femur bones were paired according to their location in the mass grave, anthropometric measurements, and DNA analysis. Out of 104 excavated femur samples (52 possible pairs) 72 were successfully matched (36 pairs). The samples were taken of the cortical bone from the upper shaft femur (under the minor trochanter), from equal femur positions of the right and the left femur bones. The average fragment length was 4.59 cm. BMD was determined on these isolated fragments.

2.2. Bone mineral density (BMD) determination

Bone density scanning (DXA scan) was performed on 72 femoral bone samples (36 pairs) at the Department of Endocrinology, University Hospital Center Split. The study was approved by the Ethical Committee of the University of Split School of Medicine, Split. BMD was measured using Hologic QDR 4500 C (S/N 48034; Bedford, MA 01730, USA) Bone Densitometer, and results were expressed as BMD (g/cm^2). Femur fragments of specific appearance and size were aligned in series of four (Fig. 1) in order to reproduce lumbar spine. Femur samples were scanned with Caucasian men normative programme (weight 75 kg, height 175 cm), followed by the analysis with Lumbar spine programme.

2.3. Grouping of the samples from DXA results

After *the bone* mineral density measurement with *DXA*, chemical analysis was performed to quantify calcium and phosphorus content in bone samples, and their mutual ratios. DXA results showed differences in BMD values between left and right femur fragments of the same person. Following the criteria of congruence obtained by densitometry, 20 femoral pairs of bones were chemically analyzed. Samples for chemical analysis were divided in two groups: fragments with the smallest BMD difference (10 matching pairs, Group 1) and fragments with the biggest BMD difference (10 matching pairs, Group 2) (Table 1).

2.4. Chemicals

Reagents used for the extraction and measurement such as standard metal solutions were of suprapur quality (Merck, Darmstadt, Germany). Standard solutions were prepared in range of expected concentration values.

2.5. Sample preparation

After the DXA measurements, compact bone pieces from femur fragments were crushed into small fragments using razor blades and stored in sterile polypropylene tubes at −20 °C until analyzed. After drying to a constant weight, the samples were washed in 6 ml of 65% nitric acid (HNO_3) overnight, washed in distilled water, and dried at the room temperature [14,15]. 0.5 g of the sample was placed in a teflon-TFM vessel, 65% nitric acid and hydrogen peroxide wet-ashed was added to the sample, and digested in automated (temperature regulated) microwave digestion unit (CEM, USA Model Mars 5 with 1600 W power). Digested samples were diluted with deionized water, and metal content was quantitatively determined. The same analysis was repeated twice using samples from different parts of the same femur fragment.

2.6. Calcium and phosphorus determination

Concentrations of elemental calcium (Ca) were determined with aModel AAS vario 6 FAAS atomic absorption spectrometer (AnalytikJena AG) in flame mode. Phosphorus was determined using UV/VIS spectrometer (model Lambda 25, Perkin Elmer, Waltham, MA USA) double beam on 650 nm wave lengths. Tungsten lamp was used as a source with 1 nm slit.

2.7. Soil analysis

The influence of *diagenesis* on calcium and phosphorus content in bone samples was determined through metal content and pH value in soil samples collected from the burial site during the excavations. Soil sample analysis (pH value in the 5% water solution of soil and quantitative Ca and P analysis) was carried out to eliminate the impact of the soil on bone contamination. To compare soil composition samples should be taken in vicinity of the bones, and from the wider area of the exhumation. Two samples were taken per each location: the first sample was from the skeletal remains level and the second sample was 20 cm below skeletal remains. Samples were prepared by microwave digestion in the same manner as the bone samples (CEM Corporation), and subjected to quantitative analysis on Atomic Absorption Spectrometer (AAS).

2.8. Method validation

Certified Standard Reference Material SRM-2710a Montana I Soil, from the National Institute of Standards and Technology (NIST, USA) was used to verify the method (National Institute of Standard and Technology, 2009). The reproducibility of sample preparation and analysis (for five times) was expressed as relative standard deviation. All the values of relative standard deviation (coefficient of variation) were less than 10%, indicating good preci-

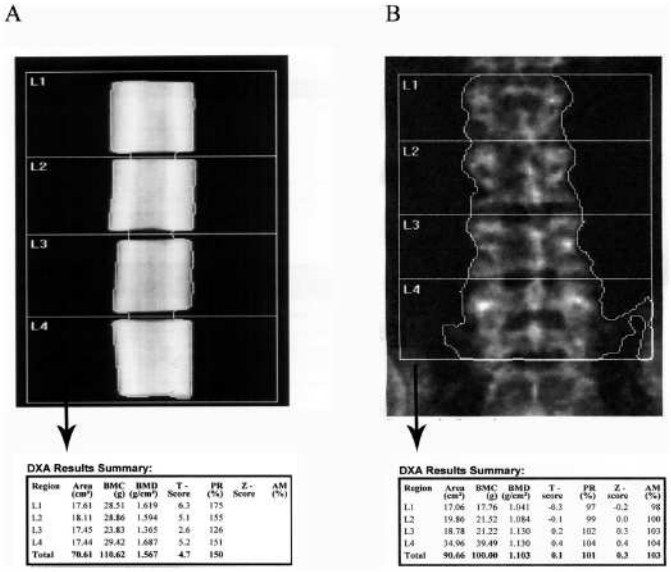


Fig. 1. DXA scanning of femur fragments. Series of four femur fragments (A) were aligned to stimulate lumbar spine (B) in order to use Lumbar spine programme for the analysis.

Table 1
DXA and chemical analysis of skeletal remains (BMD, BMD deviation, content of Ca and P, and Ca/P ratio).

Group 1*							Group 2**				
Sample No.	Femur fragment	BMD g/cm^2	BMD deviation* (%)	%Ca	%P	Ca/P ratio	BMD g/cm^2	BMD deviation* (%)	%Ca	%P	Ca/P ratio
1	L	1.316	0.15	29.12	10.22	2.849	1.409	5.04	26.47	10.71	2.472
	R	1.314		27.26	10.27	2.654	1.480		27.42	10.51	2.609
2	L	1.521	0.53	30.45	11.31	2.692	1.412	5.06	28.43	11.56	2.459
	R	1.513		29.47	10.8	2.729	1.344		29.17	11.58	2.518
3	L	1.563	0.58	26.01	10.08	2.580	1.776	5.9	25.75	9.88	2.606
	R	1.554		24.6	9.04	2.721	1.677		23.87	10.05	2.374
4	L	1.297	0.77	28.09	10.36	2.711	1.382	5.93	24.13	11.57	2.086
	R	1.307		29.58	10.53	2.809	1.464		25.77	10.5	2.454
5	L	1.741	0.93	27.41	12.24	2.239	1.575	6.35	27.82	11.82	2.354
	R	1.725		25.31	11.9	2.127	1.675		25.6	11.3	2.265
6	L	1.456	0.96	26.48	11.58	2.287	1.394	8.11	28.83	13.43	2.146
	R	1.470		29.96	11.47	2.612	1.507		27.51	11.41	2.411
7	L	1.439	1.05	30.77	12.06	2.551	1.248	10.26	27.98	11.7	2.392
	R	1.424		26.37	9.71	2.716	1.376		31.46	11.5	2.737
8	L	1.299	1.56	30.12	10.63	2.833	1.387	11.41	25.5	10.09	2.528
	R	1.279		31.97	12.62	2.533	1.254		25.43	9.68	2.627
9	L	1.594	1.57	27.59	10.68	2.593	1.396	15.26	29.3	10.8	2.713
	R	1.619		26.09	10.91	2.391	1.609		30.01	11.16	2.688
10	L	1.338	1.64	26.64	9.68	2.751	1.531	16.85	29.68	11.17	2.656
	R	1.360		26.2	9.9	2.646	1.789		30.89	11.35	2.722
Mean		1.456	0.974	27.975	10.800	2.601	1.484	9.017	27.551	11.089	2.491
Std. deviation		0.144	0.497	2.067	0.954	0.200	0.157	4.298	2.185	0.856	0.187
Minimum		1.279	0.15*	24.60	9.04	2.127	1.248	5.04**	23.87	9.68	2.086
Maximum		1.741	1.64*	31.97	12.52	2.849	1.789	16.85**	31.46	13.43	2.737

* Deviation of body mass density between left (L) and right (R) femur fragments (Group 1) is less or equal to 5%.
** Deviation of body mass density between left (L) and right (R) femur fragments (Group 2) is above 5%.

sion of measurement. Effect of matrix and chemical interference was estimated by percentage recovery of the amount guaranteed by SRM (94.6% for calcium and 103.8% for phosphorus). The detection limit for calcium was 0.1 mg/L and for phosphorus was 0.06 mg/L.

2.9. Statistical analysis

The significance of differences between values was calculated using SPSS software 11.03 for Windows and MS Office Excel 2010 packages. T-test, Pearson and Partial correlation were used

Table 2
Results of BMD, calcium and phosphorus content, calcium-phosphorus ratio in bones, and two soil samples from two locations (first soil sample is from the level of skeletal remains and second sample is 20 cm below skeletal remains).

Parameters		Location 1		Location 2	
		Bone samples (N = 22)	Soil samples (mean value)	Bone samples (N = 18)	Soil samples
BMD (g/cm^2)	Minimum	1.248		1.245	
	Maximum	1.789		1.776	
	Median	1.409		1.517	
Ca (%)	Minimum	25.310		23.87	
	Maximum	31.969		30.77	
	Median	28.975	13.3	26.050	12.5
P (%)	Minimum	10.220		9.040	
	Maximum	13.430		12.060	
	Median	11.380	0.068	10.295	0.073
Ca/P ratio	Minimum	2.127		2.086	
	Maximum	2.849		2.751	
	Median	2.611		2.587	
pH (5%-water solution)			6.8		7.5

Table 3
Correlation coefficients for groups I and II (N = 20 in each group).

Pairs	Group I		Group II	
	Correlation coefficients of pairs (r)	*P*	Correlation coefficients of pairs (r)	*P*
BMD & P	0.265	0.259	−0.155	0.514
BMD & Ca	−0.447	0.048	−0.011	0.964
	Partial correlation coefficient			
	Ca		P	
Ca	1.0000		0.4965† (*P* = 0.031)	
P	0.7365* (*P* ⩽ 0.05)		1.0000	

* Strongly correlation.
† Significant correlation.

to calculate correlation between measurements. For all statistical tests significance level of 95% ($P \leqslant 0.05$) was used.

3. Results

Based on BMD results, 20 pair of bones (from the left and the right femur) of middle aged men were divided into 2 groups, and afterwards chemically analyzed. The values of calcium mass (in percent) varied between 23.87 and 31.97%, while the phosphorus mass ranged from 9.04 to 13.43%. The deviation of phosphorus concentration in left and right femoral pair fragments has been lower than the deviation of calcium concentration in the same samples (Table 1).

The results were compared in regard to calcium and phosphorus concentration in soil samples, as well as the pH values of the soil. The soil sample taken from location 1 had lower pH value, and was correlated with higher calcium concentration. The soil acidity did not correlate with phosphorus concentration (Table 2). The correlation of measurement results was analyzed by the pair T-test.

In comparison of BMD and phosphorus content, weak correlation was found with samples taken from Group I ($r = 0.265$, $P = 0.259$). Negative correlation was identified for the samples taken from Group II ($r = -0.155$, $P = 0.514$). In comparison of BMD and calcium content, no correlation was found for both groups. The correlation factor for samples from Group I was −0.447 ($P = 0.048$) and for Group II the factor was −0.011 ($P = 0.964$) (Table 3).

Partial correlation was performed to determine BMD dependency on joined calcium and phosphorus concentration. Significant correlation was demonstrated between BMD and joined calcium and phosphorus concentration in both Groups tested (especially for Group I, $r = 0.7365$, $P \leqslant 0.05$) (Table 3).

4. Discussion

This study was performed to verify the concordance amongst DXA scanning of bone mineral density (BMD) and chemically analyzed mineral content of archaeological samples (femoral skeleton bone). Bone mineral density varies among bones, and even between different parts of the same bone [16]. DXA calculates the amount of hydroxyapatite in bones, which is mainly consistent from calcium and phosphorus. The ratio of Ca/P in hydroxyapatite mineral *in vitro* is 1.5–1.67 [17], while the ratio of Ca/P in hydroxyapatite of human bones does not vary, and amounts 2.2 [8,18].

So far, no one compared Ca/P ratio in skeleton samples. Few studies determined Ca/P ratio from bone samples taken 24 h after death [18,19], and the result were consistent with Ca/P ratio of living human. Also, in Croatia, DXA method is due to different reasons (mainly financial), still used as a diagnostic tool only, rather than a useful method in forensic medicine; hopefully this paper will stimulate the use of that method in such aspect as well as other research, too.

Studied samples must be carefully selected to make the results representative. Tapper et al. analyzed metals in archaeological bones of children (6th–8th century) from Ficiane region in Italy,

and found low content of calcium [20], presumably as a result of trabecular bone being analyzed instead of recommended cortical. In our study, the best preserved fragments of cortical femoral bone were subjected to analysis.

Interpretation of the element concentrations evaluated from excavated bone material must be in context with the soil analysis and grave goods. During its stay in soil, a bone is subjected to the process of diagenesis [21,22]. One of the first modifications during bone diagenesis is dissolution of minerals and their exchange with the surrounding soil [23]. Our exhumation site was located on the small island (0.07 km^2) surrounded by the Adriatic Sea. The island is consisted of *terra rossa* soil, and dolomite and limestone rocks and minerals [24]. The soil on exhumation site was close to the pine forest and had neutral to slightly acidic pH values. The values of phosphorus mass percent in the bone samples are no different from the results published in earlier studies [25–28], while the values of calcium are significantly higher than expected. Location 1 had lower pH of soil, corresponding to slightly higher calcium values in bones (Table 2). Acidic conditions are favourable for better dissolution of calcium. The pH values of soil from location 2 were slightly alkaline, resulting in lower bone calcium values compared with location 1. The ratio of calcium and phosphorus is consequently higher, indicating soil influence on archaeological bones.

The correlation between BMD and the mass percent of calcium has not been proven in examined groups, and only a weak correlation between the BMD and the mass percent of phosphorus was identified only in Group 1. The results obtained in this study do not support hypothesis that BMD measured by DXA scan will have positive correlation with chemically determined concentrations of calcium and phosphorus in bones, especially for calcium. Since the soil of exhumation site is rich in calcium, and calcium of biological origin cannot be distinguished from calcium found in the soil, increased values of the calcium mass could be explained by diagenesis.

Our findings indicate that interpretation of results obtained only by DXA scan from skeleton remains can be misleading since without Ca/P ratio values there is no certainty of what is really being measured. Namely, DXA scan cannot distinguish calcium (or phosphorus) content developed during lifetime from calcium built in bones during diagenesis.

5. Conclusion

The results of this study do not support our initial hypothesis that BMD and mineral content (calcium and phosphorus) measured by DXA scanning is positively correlated with the concentrations of calcium and phosphorus measured by chemical analysis. Namely, while the concentrations of phosphorus were according to expectations, the values for calcium were significantly higher, indicating possible diagenesis effect on skeletal remains. Since this is the first study correlating BMD and Ca/P ratio of skeleton remains, further research should be undertaken in order to expand our knowledge. Our findings are also in accordance with previous findings regarding the advantages and use of DXA method in forensic medicine.

Dual-energy X-ray absorptiometry (DXA) scanning is a gold standard for BMD measurement in living persons, but our results suggest the necessity of chemical determination of Ca/P ratio when analysing skeleton remains.

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4. ZNANSTVENI DOPRINOS OBJEDINJENIH RADOVA

Rezultati dobiveni u ovom istraživanju ne podržavaju hipotezu da BMD mjeren denzitometrijom ima pozitivnu korelaciju s kemijski određenim koncentracijama kalcija i fosfora u kostima, što je posebice izraženo za kalcij. Zbog toga tumačenje rezultata dobivenih samo primjenom denzitometrije kod analize kosturnih ostataka može voditi pogrešnim zaključcima. Kako se denzitometrija koristi ne samo kao dijagnostička metoda za osteoporozu živih osoba, već i u arheološkim i forenzičkim ispitivanjima, naš rad predstavlja znanstveni doprinos boljem razumijevanju i vrednovanju rezultata denzitometrije na skeletnom materijalu, posebice stoga što ovakvo istraživanje nije, prema našim saznanjima, do sada objavljeno u dostupnoj literaturi. Također, predložen je vodič za postupanje s uzorcima iz masovnih grobnica, koji pretpostavlja niz mogućih faza u svrhe obrade i identifikacije žrtava kao i u znanstvene svrhe.

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PUBLIKACIJE

Radovi objavljeni u časopisima koji se indeksiraju u bazi Current Contents:

1. Borić I, Ljubković J, Sutlović D. Discovering the 60 years old secret: Identification of the World War II mass grave victims from the island of Daksa near Dubrovnik, Croatia. Croat Med J 2011; 52:327-35.
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OBITELJ

Oženjen i otac šestero djece, za sada djed jednoj unuci.

OSTALO

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